Excess Phlda2 as a mouse model of intrauterine growth restriction

Simon James Tunster

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Abstract

A small fraction of mammalian genes exhibit parent-of-origin specific monoallelic expression. They are expressed from only one allele and this is determined by modifications established in the germline. Approximately 100 imprinted genes have been identified to date. Most imprinted genes are located in discrete clusters and are controlled by shared regulatory elements. Imprinted genes play important roles in regulating embryonic and placental development, with overt growth phenotypes resulting both from loss of expression and from over-expression of imprinted genes.

The maternally expressed *Phlda2* gene has been implicated in placental development. Loss of expression leads to placentomegaly as a consequence of the disproportionate expansion of the spongiotrophoblast layer. In this study, the consequences of over-expressing *Phlda2* and the adjacent *Slc22a18* were investigated in four independent lines of transgenic mice driving incrementally increasing doses of the two genes and on two genetic backgrounds. In all cases, transgenic placentae were significantly lighter throughout gestation, which was entirely due to a reduction in the spongiotrophoblast layer. There was also a reduction in glycogen staining and a progressive mislocalisation of cells from the spongiotrophoblast layer. These phenotypes were essentially restored by restored by normalising *Phlda2* gene dosage in a single copy line.

In addition, transgenic embryos were significantly lighter than wild type littermates from E16.5 onwards and were born 13% lighter. These embryos were asymmetrically growth restricted and displayed rapid post-natal catch up growth within two weeks of birth. Adult transgenic females that had undergone embryonic growth restriction also displayed increased adiposity and reduced glucose tolerance at one year of age. These data suggest that altered expression of *Phlda2* and possibly *Slc22a18* drive IUGR and program adult disease susceptibility. Recent human studies have found an association between elevated placental *PHLDA2* and low birth weight or IUGR infants. This mouse model may thus provide a genetic tool that recapitulates a known human condition for further investigation of the fetal programming of metabolic syndrome.

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List of Abbreviations

AS	Angelman Syndrome
ATP	Adenosine triphosphate
BAC	Bacterial Artificial Chromosome
bp	base pairs
BMI	Body mass index
BWS	Beckwith Wiedemann syndrome
cDNA	Complimentary DNA
CNS	Central Nervous System
CpG	Cytosine-phosphate-guanine
CTCF	CCCTC binding factor
C_T	Cycle threshold
C-TGC	Canal-associated trophoblast giant cell
CTP	Cytidine triphosphate
CVD	Cardiovascular disease
dH_2O	Distilled H_2O
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
EtOH	Ethanol
GTP	Guanosine triphosphate
HAT	Heterodimeric amino acid transporter
H&E	Haematoxylin and Eosin
HC/AC	Head circumference to abdominal circumference ratio
HDL	High density lipoprotein
IC	Imprinting centre
ICM	Inner cell mass
ICR	Imprinting control region
IPTG	Isopropyl β-D-thiogalactopyranoside solution
IUGR	Intrauterine growth restriction
kb	Kilobases
kbp	Kilobase pairs
KO	Knockout
LOI	Loss of imprinting

MatDp	Maternal duplication
MCS	Multiple cloning site
MeOH	Methanol
MMLV	Moloney Murine Leukemia Virus
Mya	Million years ago
NTMT	NaCl, Tris-HCl, Magnesium Chloride, Tween solution
ncRNA	Non-coding RNA
PAS	Periodic acid Schiff
PatDp	Paternal duplication
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PH	Pleckstrin homology
P-TGC	Parietal trophoblast giant cells
PWS	Prader-Willi Syndrome
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RT	Reverse transcription
SOB	Super Optimal Broth
SOC	Super Optimal Broth with Catabolite Repression
SpA-TGC	Spiral-associated trophoblast giant cell
SSC	Saline sodium citrate
S-TGC	Sinusoidal trophoblast giant cell
TAE	Tris-acetate EDTA
Tg	Transgenic
TGC	Trophoblast giant cell
T_m	Melting temperature
tRNA	Transfer RNA
UPD	Uniparental disomy
UTP	Uridine triphosphate
WT	Wild type

Chapter 1:

General Introduction

1.1 Gene expression and genomic imprinting

Diploid animals possess two homologous copies of each autosomal chromosome, inheriting an entire haploid set from each parent at fertilisation. Autosomal genes are thus represented by two alleles, which can both be transcriptionally active for most genes (reviewed in Watanabe and Barlow 1996). Such biallelic expression from a diploid genome is proposed to be advantageous by protecting against the potential consequences of genetic mutations (Orr 1995; Otto and Goldstein 1992). Haploid organisms possess a single copy of each gene, with mutations potentially resulting in the complete loss of functional gene product. In a diploid organism, the same mutation will generally result in only a reduction in the amount of functional gene product, with the homologous allele remaining functional.

Despite the benefit of possessing two actively expressed alleles for each gene, several types of mammalian genes are expressed from only one allele, with the other allele rendered inactive (reviewed in Goldmit and Bergman 2004). Such monoallelically-expressed genes can be classified according to the mechanisms involved in their regulation. The most extensively studied of these is X-inactivation, which results in the random inactivation of an entire X-chromosome in somatic cells of females (reviewed in Avner and Heard 2001; Lyon 1961). This dosage compensation ensures that the expression level of X-linked genes is comparable between males and females, which possess only one X chromosome. Another mechanism that results in monoallelic expression is termed "random allelic exclusion". This method is important for ensuring the expression of a single cell surface receptor molecule in B-lymphocytes. Allelic exclusion has also been observed in the regulation of odorant receptor expression in olfactory neurones for a similar reason (Chess *et al.* 1994; Corcoran 2005; Efstratiadis 1995; Mostoslavsky *et al.* 2004). A third mechanism associated with monoallelic gene expression is "genomic imprinting". This describes the expression of imprinted genes in a parent-of-origin specific manner (see Fig 1.1) (Surani *et al.* 1984). The activity of a particular imprinted gene is always inherited through the same germline. For example, the gene encoding insulin-like growth factor 2 (*Igf2*) is expressed from the paternally inherited allele in both mice and humans (DeChiara *et al.* 1991).

Imprinted genes are often defined as genes that exhibit parent-of-origin specific, monoallelic expression (Ferguson-Smith and Surani 2001; Mochizuki *et al.* 1996; Pfeifer 2000; Reik and Walter 2001b). However, some imprinted genes do not exhibit the strict monoallelic expression pattern suggested by this definition, and instead display preferential allelic expression (Schulz *et al.* 2006). A recent review of the literature concluded that of 27 maternally expressed imprinted genes, 21 displayed preferential expression of the maternal allele, whereas 18 of 23 paternally expressed genes exhibit strict monoallelic expression (Khatib 2007). Furthermore, some imprinted genes are expressed monoallelically in all cell types, whereas others exhibit monoallelic expression in only a subset of tissues. For instance, the gene encoding the mouse insulin-like growth factor 2 receptor (*Igf2r*) exhibits monoallelic expression in peripheral tissues, such as liver and kidney, but is expressed biallelically in the central nervous system (CNS) (Hu *et al.* 1998). Thus an imprinted gene is more accurately defined as a gene that exhibits differential expression of parental alleles in one or more tissues (Moore 2001).

Monoallelic expression of imprinted genes eliminates the protection afforded by the presence of two active alleles. Deletions affecting the active allele of an imprinted gene are often associated with human disease. The human neurological disorders Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are characterised by distinct clinical symptoms, but are both associated with deletion of the chromosomal region 15q11-13. This region contains several imprinted genes, including the paternally expressed *SNRPN* and *NDN* and the maternally expressed *UBE3A* (reviewed in Glenn *et al.* 1997). Paternal inheritance of the q11-13 deletion can cause PWS implicating loss of *SNRPN* and *NDN* in PWS (Jay *et al.* 1997; Ozcelik *et al.* 1992). Inheriting the deletion on the maternally inherited chromosome is causative of AS implicating loss of *UBE3A* expression in AS (Kishino *et al.* 1997; Knoll *et al.* 1989; Nicholls *et al.* 1989). Loss of imprinting (LOI) is also associated with human disease, and is particularly prevalent in various cancers (reviewed in Falls *et al.* 1999; Jelinic and Shaw 2007; Lalande 1997; and Rainier *et al.* 1993).

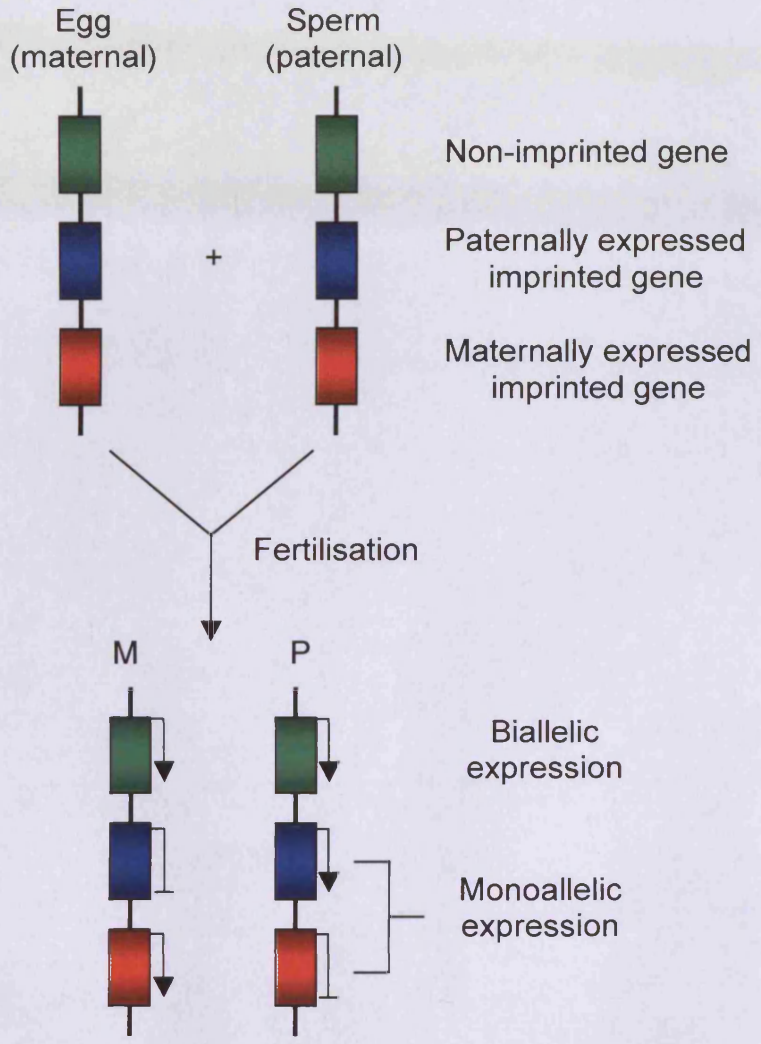


Figure 1.1: Inheritance and expression of biallelic and imprinted genes

Imprinted genes are inherited in accordance with the rules of Mendelian genetics, with one allele inherited from each parent. Expression of imprinted genes however does not conform to the rules of Mendelian genetics, with one parental allele exhibiting preferential expression over the other. Expression of most autosomal genes is biallelic; both alleles are transcribed equally (green). Imprinted genes however, are expressed in a parent-of-origin dependent manner, with preferential expression resulting from either the paternal allele (blue) (e.g. *Igf2*) or the maternal allele (red) (e.g. *Igf2r*).

In contrast to X-inactivation in embryonic cells and allelic exclusion, in which the repressed allele is randomly selected, imprinting is a parent-of-origin specific process, with the actively expressed allele expressed from only the maternal or paternal chromosome. This method of gene regulation requires an "imprint", by which the parental origin of each allele is identified. The differential methylation of a transgene arising from passage through the paternal and maternal germline in mice suggested a role for DNA methylation in the imprinting process (Hadchouel *et al.* 1987; Reik *et al.* 1987; Sapienza *et al.* 1987; Swain *et al.* 1987). This role was subsequently confirmed in mice deficient for DNA methyltransferase, which exhibit LOI of several genes due to a lack of CpG dinucleotide methylation (Hata *et al.* 2002; Kaneda *et al.* 2004; Li *et al.* 1993; Okano *et al.* 1999).

Imprinted loci acquire methylation on passage through either the maternal or paternal germline, generating differentially methylated regions (DMRs), which are now a recognised characteristic of imprinted genes (Constância *et al.* 1998; Mann 2001; Smith *et al.* 2003). The maternal genome acquires the majority of imprinting-associated methylation, although this does not necessarily correspond with gene repression (Delaval and Feil 2004; Reik and Walter 2001a, b). The methylation of imprinted genes acts to mark the parental alleles in the germline (Surani 1998). The "interpretation" of this imprint is performed by more complex mechanisms, which include histone modifications and additional DNA methylation that ensures differential allelic expression (reviewed in Reik and Walter 2001b; and Surani 1998).

The methylation imprint is established in the germline (Tucker *et al.* 1996), the time at which the two parental genomes exist in isolation, enabling a differential methylation pattern to be established. Methylated regions are established by members of the Dnmt3 family of methyltransferases (Kato *et al.* 2007), including Dnmt3a (Kaneda *et al.* 2004), Dnmt3b (Okano *et al.* 1999) and Dnmt3l (Hata *et al.* 2002). These methylation marks must be maintained through successive cycles of cell division in somatic cells, and during the global demethylation of the parental genomes following fertilisation. This imprint maintenance is performed by the methyltransferase Dnmt1 (Branco *et al.* 2008; Li *et al.* 1993), which specifically recognises and methylates the newly synthesised strand during DNA replication (Bestor 2000; Branco *et al.* 2008; Hirasawa *et al.*

2008). Methylation of imprinted loci is erased in the germline and re-established according to the individual's sex, thus completing a cycle of establishment, maintenance, erasure and re-establishment, as depicted in Fig 1.2 (reviewed in Constância *et al.* 1998; Reik *et al.* 2001; and Szabo and Mann 1995).

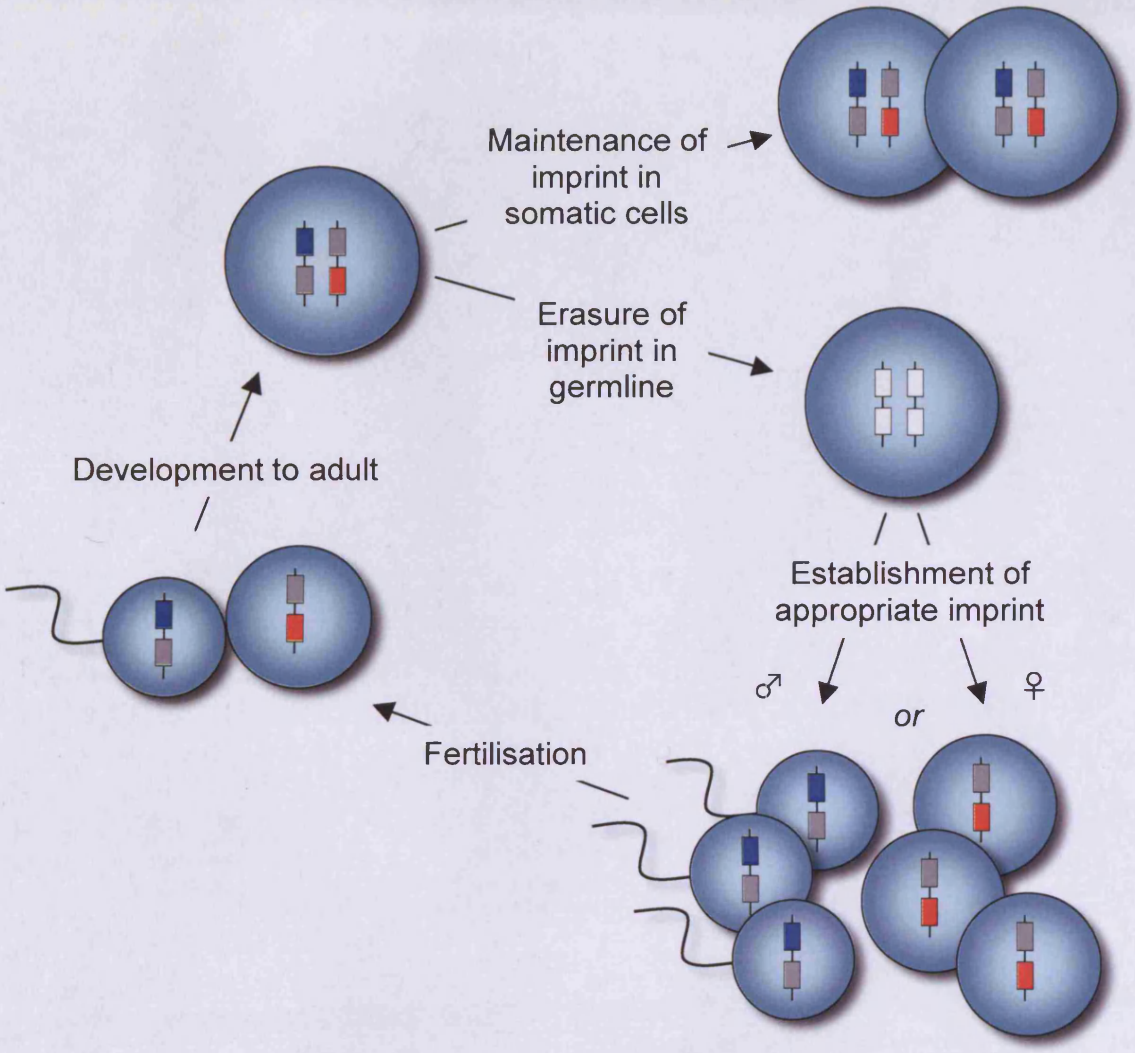


Figure 1.2: Dynamic imprinting

Imprinted genes are identified by differential methylation of the two parental alleles established in the germline. This pattern of methylation is maintained in somatic cells by the DNA methyltransferase Dnmt1. In subsequent generations, the imprint is erased in germline cells prior to re-establishment of the appropriate methylation mark according to the sex of the individual. At fertilisation, two haploid genomes with the appropriate methylation marks combine to give a diploid genome, with the correct dosage of imprinted genes. Blue = paternally active, red = maternally active, grey = inactive allele.

1.2 Parthenogenesis and genomic imprinting

Genomic imprinting was first identified in the early 1980s from investigations into the failure of parthenogenesis (embryogenesis of an unfertilised oocyte) in mice (Mann and Lovell-Badge 1984; McGrath and Solter 1984; Surani *et al.* 1984). Parthenogenetic reproduction is common in invertebrates, occurs more rarely in vertebrate species such as reptiles, birds and fish, yet has not been reported to occur naturally in mammals (Chapman *et al.* 2007; Kaufman *et al.* 1977; Rougier and Werb 2001; Watts *et al.* 2006). Mouse oocytes in which embryogenesis was experimentally induced were not viable. These parthenogenetic embryos generally died soon after implantation at embryonic day 4.5 (E4.5), although a few developed to the 25-somite stage (~E10.5). Parthenogenetic embryos that survived to these advanced developmental stages appeared growth restricted but otherwise relatively normal, in contrast to extraembryonic development, which was severely restricted and disorganised (Kaufman *et al.* 1977; Sturm *et al.* 1994; Tarkowski *et al.* 1970).

The failure of parthenogenetic embryos to survive to term was initially suggested to be due to either the absence of a cytoplasmic contribution from sperm or a consequence of homozygosity of recessive lethal mutations resulting in the uniparental genome (Graham 1974). The need for a cytoplasmic contribution from sperm was excluded following the generation of viable embryos from normal genetic material transplanted into enucleated parthenogenetic eggs (Mann and Lovell-Badge 1984; Surani *et al.* 1984). Furthermore, Surani and colleagues reported that haploid parthenogenetic eggs that receive a second female pronucleus fail to develop to term, whereas those that received a male pronucleus were viable (Surani *et al.* 1984). The viability of a heterozygous bi-parental genome compared with the developmental failure of a uniparental heterozygous maternal genome led to the conclusion that the both parental genomes are essential for embryonic development (Surani *et al.* 1984). Support for this came from the observation that androgenetic conceptuses, consisting of two paternal genomes, also fail to support normal embryogenesis (Barton *et al.* 1984; McGrath and Solter 1984)

Gynogenetic and parthenogenetic embryos exhibit a similar fate, generating relatively normal, albeit growth restricted embryos that die soon after

implantation, with approximately 20% surviving until around E10.5. The associated extraembryonic tissue is almost non-existent, and may be a contributing factor to embryonic lethality. Androgenetic embryos exhibit a contrasting phenotype with severe developmental retardation of the embryo and death at around E8.5, with an abundance hyperplastic extraembryonic tissue (Barton *et al.* 1984; Kaufman *et al.* 1977; McGrath and Solter 1984; Surani *et al.* 1986). Gynogenetic, parthenogenetic and androgenetic embryogenesis is summarised in Table 1.1. The reciprocal phenotypes associated with gynogenetic/parthenogenetic and androgenetic conceptuses suggested complementary roles for the two parental genomes in the development of the embryo and placenta. These non-equivalent functions of the parental genomes were proposed to be the result of genomic imprinting, whereby specific genes are expressed from only one parental genome (Surani *et al.* 1984). This theory was confirmed by the findings that both *Igf2* and *Igf2r* exhibit parental specific patterns of expression in the mouse. The paternally expressed *Igf2* gene encodes a potent growth-promoting factor (DeChiara *et al.* 1990), whereas the maternally expressed *Igf2r* gene encodes a receptor that binds and sequesters Igf2, thus acting in a growth inhibitory role (Barlow *et al.* 1991). In uniparental embryos, imprinted gene expression is disrupted such that some genes are expressed at twice the normal level whereas others are not expressed at all. For example, in androgenetic embryos, the paternally expressed *Igf2* gene is expressed at two-fold normal levels, whereas expression of the maternally expressed *Igf2r* is completely ablated (Walsh *et al.* 1994).

The substantial extraembryonic tissue development in androgenetic embryos suggested that genes required for placental development were expressed only from the paternal genome, whereas those controlling stages of embryonic development were expressed from the maternal genome (Barton *et al.* 1984). Later experiments using chimeric mice generated from a mixture of parthenogenetic/gynogenetic and androgenetic cells revealed a striking pattern of segregation of the two cell types, with trophoblast tissue tending to consist of androgenetic cells and the embryo being formed largely from parthenogenetic/gynogenetic cells (Surani *et al.* 1987; Thomson and Solter 1988). Such chimeras still failed to survive to term indicating that there was an incomplete complement of gene expression, and that some placental-specific

genes are maternally expressed and some embryo-specific genes are paternally expressed.

	Genomic content	Embryonic development	Placental development
Parthenogenetic	Two maternal genomes (homozygous)	Growth retarded Death by ~E10.5	Poor
Gynogenetic	Two maternal genomes (heterozygous)	Growth retarded Death by ~E10.5	Poor
Androgenetic	Two paternal genomes (heterozygous)	Disorganised Death by ~E8.5	Hyperplastic

Table 1.1: Developmental phenotypes of parthenogenetic, gynogenetic and androgenetic conceptuses

Development of the embryo is relatively normal, although delayed where two maternal genomes are present; extraembryonic development is severely disrupted. In androgenetic conceptuses, extraembryonic tissue develops relatively normally, however embryonic development is severely affected.

The creation of a parthenogenetic mouse that survived to adulthood and gave birth to viable offspring was recently reported. However, this was only achieved following genetic manipulation to yield monoallelic expression of the *H19* domain (Kono *et al.* 2004; Kono *et al.* 2002). Correct dosage of the growth factor *Igf2* thus appears to be at least one critical factor for successful embryogenesis. Furthermore, only two of nearly 400 such conceptuses that were implanted survived, with one yielding viable offspring, indicating that expression levels of other imprinted genes may also be a factor in determining embryonic survival (Kono 2006; Kono *et al.* 2004). Normalised expression of several imprinted genes independent of the *H19* region was observed in these parthenogenetic embryos. The authors postulate that corrected expression of the maternally expressed *Meg3* (*Dlk2*) and the paternally expressed *Dlk1* genes in some parthenogenetic conceptuses may contribute to enhanced viability. However, it remains unclear how expression of these unrelated genes is normalised in such parthenogenetic conceptuses (Kono *et al.* 2004).

1.3 *Imprinted gene clusters and their regulation*

The reciprocal growth phenotypes of gynogenetic/parthenogenetic and androgenetic embryos revealed that the two parental genomes perform complementary roles during development potentially as a consequence of parental imprinting of the genome (Surani *et al.* 1984). The initial nuclear transplantation experiments however did not yield any indication as to the extent or location of imprinting in the genome. Phenotypes observed in mice with maternal or paternal duplication (MatDp or PatDp respectively) of specific chromosomal regions, led to the identification of a number of imprinted regions in mice. Conceptuses with MatDp or PatDp for chromosomal regions including 2, 6, 7, 11 and 17 resulted in embryonic and/or placental growth defects, indicating parental imprinting of these regions (Cattanach and Beechey 1990). For instance, MatDp of mouse distal chromosome 7 results in small placentae and growth restricted embryos that die at ~E16.5 (Searle and Beechey 1990). PatDp of the same region is associated with loss of the spongiotrophoblast layer from the placenta and embryonic death at approximately E10.5 (McLaughlin *et al.* 1996; Searle and Beechey 1990). In contrast, conceptuses with MatDp or PatDp of chromosomes 1, 4, 5, 9, 13, 14 or 15 are phenotypically normal, suggesting the absence of imprinting of these chromosomes (Cattanach and Kirk 1985).

The first imprinted genes to be described were discovered from the phenotypic analysis of knockout mouse models. The growth deficiency associated with paternal transmission of an *Igf2* null allele was not observed when this same allele was transmitted through the maternal germline (DeChiara *et al.* 1990; DeChiara *et al.* 1991). Likewise, the embryonic lethality associated with maternal deletion of *Igf2r* was not observed in embryos with a paternal deletion of this gene (Barlow *et al.* 1991). Analysis of mouse uniparental disomy (UPD) phenotypes led to the identification of 11 imprinted regions located on seven chromosomes in the mouse (reviewed in Cattanach and Beechey 1997; Cattanach and Kirk 1985). However, identification of imprinted genes in this manner assumes that inheritance of both copies of an imprinted gene from one parent will yield overt embryonic or placental defects. Further to the analysis of UPD growth phenotypes, additional approaches have been undertaken to identify imprinted genes within the genome (reviewed in Maeda and Hayashizaki 2006;

Oakey and Beechey 2002; and Peters and Beechey 2004). One such approach is to specifically search for DMRs, which are often associated with imprinted regions (reviewed in Constância *et al.* 1998). The paternally expressed genes *Nap1l5*, *Peg13* and *Slc38a4* were identified utilising this technique (Smith *et al.* 2003). Another approach is to compare the expression profiles of PatDp and MatDp tissue by "subtraction hybridisation". The expression of a maternally expressed gene would be two-fold wild type levels in MatDp and completely lost in PatDp, whereas the opposite would be true for paternally expressed genes. Biallelically expressed genes would show no change in expression levels. Several imprinted genes have been identified in this way, including the paternally expressed genes *Peg1* and *Peg5* and the maternally expressed *Grb10* (Kagitani *et al.* 1997; Kaneko-Ishino *et al.* 1995; Miyoshi *et al.* 1998). A similar approach utilising microarray analysis of uniparental disomies identified imprinted expression of *Dhcr7*, *Th* and *Ampd3* in the mouse placenta (Schulz *et al.* 2006).

To date, imprinted genes have been identified on twelve different chromosomes in the mouse, including chromosomes 9, 14 and 15, which did not result in growth phenotypes when inherited from only one parent (Cattanach and Kirk 1985). The position of imprinted genes identified through a variety of approaches have been collated to generate an "imprinting map" of the mouse genome, depicting the chromosomal location of all known imprinted genes (see Fig 1.3) (Cattanach *et al.* 2004; Cattanach and Kirk 1985). A total of approximately 100 imprinted genes have been identified to date, of which approximately one quarter are non-coding RNA molecules (ncRNA) (Morison *et al.* 2005; Peters and Beechey 2004). Estimates for the total number of imprinted genes range from around 100 to over 600, with the most realistic expectations generally considered to be at the lower end of this range (Barlow 1995; Luedi *et al.* 2005; Morison *et al.* 2005; Wang *et al.* 2008).

The imprinting map revealed a tendency for imprinted genes to localise in discrete clusters within the genome. Approximately 80% of imprinted genes are physically linked in these "imprinted regions" or "imprinted domains" (Reik and Walter 2001b), three of which are located on mouse chromosome 7 (see Fig 1.3). The clustered organisation of imprinted genes suggested the co-ordinated control of imprinted expression. Investigations using mouse models demonstrate that *cis*-acting sequences known as imprinting control regions (ICRs) or imprinting

centres (ICs) establish domain-wide allele-specific gene expression (Edwards and Ferguson-Smith 2007; Lewis and Reik 2006; Spahn and Barlow 2003). ICs acquire germline-specific DNA methylation, with mutation or deletion of these regions resulting in LOI of surrounding genes (Lewis and Reik 2006).

Imprinted clusters generally consist of a number of protein-coding genes in addition to at least one ncRNA gene (Edwards and Ferguson-Smith 2007; Pauler *et al.* 2007). The ncRNA is generally expressed from the domain on which the IC is unmethylated, whereas the protein-coding genes tend to be expressed from the domain on which the IC is methylated (O'Neill 2005; Pauler and Barlow 2006; Pauler *et al.* 2007; Spahn and Barlow 2003). In contrast to situations such as the direct silencing of transposable elements by DNA methylation (Bird 1992), imprinted gene repression is instead often associated with transcription of the ncRNA molecule in *cis*. Indeed, two mechanisms have been described that are involved in the control of imprinted domains. One mechanism involves the expression of the ncRNA transcript from one parental allele, which acts in *cis* to prevent the expression of adjacent imprinted genes. Methylation of the other parental allele silences expression of the ncRNA, thus permitting expression of the protein-coding genes (reviewed in O'Neill 2005). The "insulator" or "enhancer blocking" mechanism involves competition for shared enhancer sequences, and is exemplified by the *Igf2/H19* locus. Binding of the zinc-finger protein CTCF to the unmethylated maternal allele prevents interaction of the *Igf2* promoter with the downstream enhancer, thus repressing the maternal *Igf2* allele. Methylation of the paternal allele prevents CTCF binding and thus enables *Igf2* to interact with the promoter (see section 1.7.1 for further detail).

The genetic organisation of imprinted domains is generally well conserved between humans and mice, however the imprinting status of some genes is not always maintained between the species (Khatib *et al.* 2007; Morison *et al.* 2005). For instance, the organisation of mouse distal 7 and the syntenic human region on chromosome 11p15.5 are generally well conserved, with most genes exhibiting the same pattern of imprinting in both species (Paulsen *et al.* 1998; Paulsen *et al.* 2000). Two genes for which imprinting is not conserved are *Tssc4/TSSC4*, which is imprinted in the mouse but not human, and *Ltrpc5/LTRPC5*, which is imprinted in human but not mouse (Paulsen *et al.* 2000).

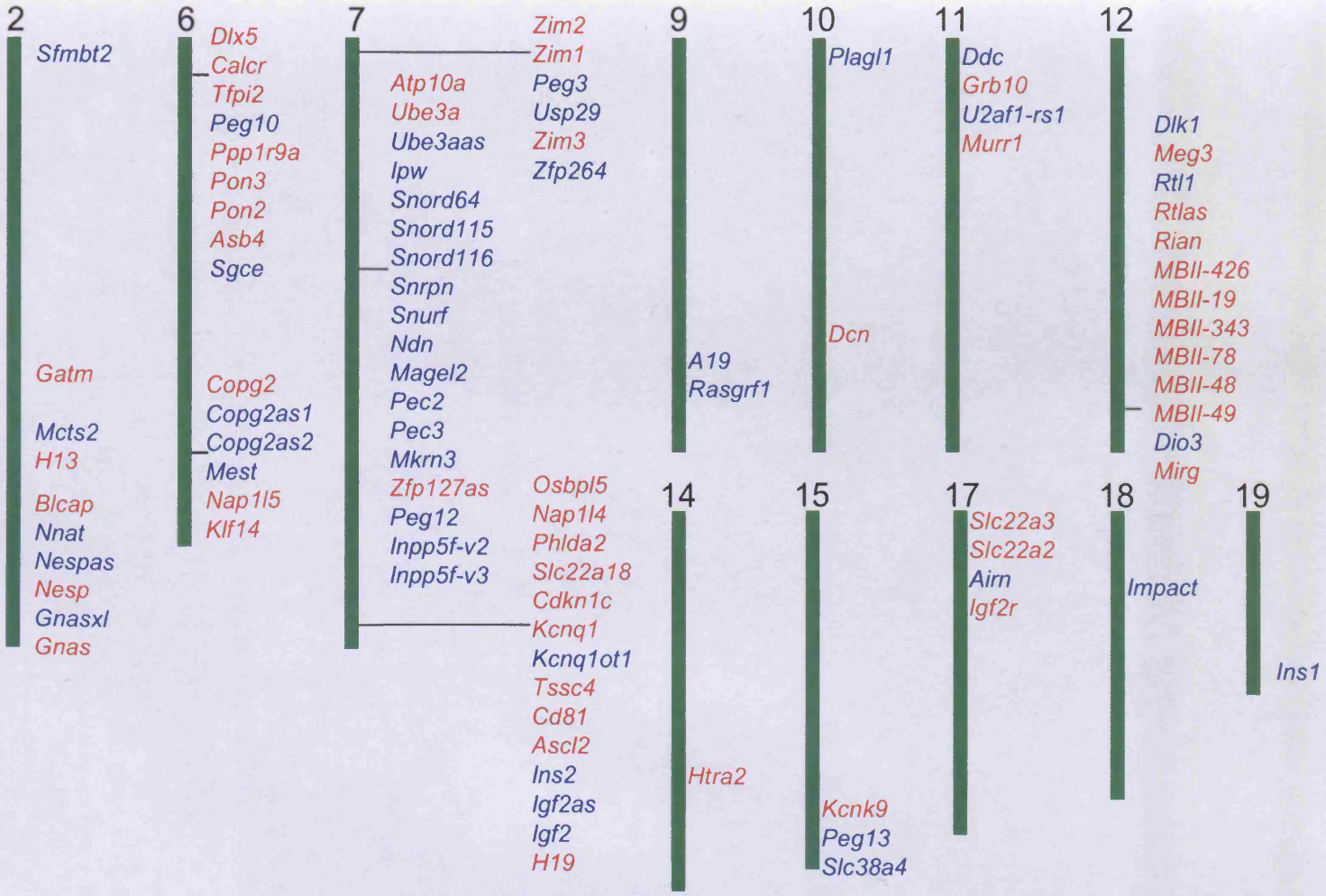


Figure 1.3: Mouse imprinting map

Imprinted genes are not evenly dispersed throughout the genome, but tend to be found in clusters. Blue = paternally expressed; red = maternally expressed; black = approximate centre of domain.
Adapted from: http://www.har.mrc.ac.uk/research/genomic_imprinting/maps.html, and updated using information from http://www.geneimprint.com/site/genes-by-species.Mus+musculus

1.4 *Imprinted genes in growth and development*

The developmental abnormalities associated with gynogenetic, parthenogenetic and androgenetic conceptuses clearly demonstrated a role for imprinted genes in the regulation of growth and development of the embryo and placenta. Indeed, the majority of protein coding imprinted genes for which a function has been assigned are expressed in the embryo and/or the placenta (reviewed in Coan *et al.* 2005a; Isles and Holland 2005; Morison and Reeve 1998; Reik *et al.* 2003; and Tycko and Morison 2002). However, imprinted genes have recently been identified that are involved in neonatal growth, namely the maternally expressed *Gnas* and *Atp10a* and the paternally expressed *Gnasxl* and *Rasgrf1* (Dhar *et al.* 2004; Font de Mora *et al.* 2003; Xie *et al.* 2006; Yu *et al.* 2000).

In mice, the majority of imprinted genes are expressed in the placenta where they sometimes exhibit a more stringent pattern of allele-specific expression compared to embryonic tissues (Coan *et al.* 2005a; Tycko 2006). Imprinted genes are involved in numerous aspects of placental development and function, including the regulation trophoblast differentiation and nutrient transfer (reviewed in Coan *et al.* 2005a). Most imprinted genes that act in the placenta control aspects of placental development, for instance the maternally expressed *Ascl2* (*achaete-scute complex homolog 2*; formerly *Mash2*) is essential for the differentiation of spongiotrophoblast cells. Interestingly, loss of the paternally expressed *Peg10* also causes a loss of spongiotrophoblast although giant cell number was unaffected; *Peg10* null embryos die by E10.5 (Ono *et al.* 2006). Additionally, some placental nutrient transporters are encoded by imprinted genes, including the maternally expressed *Slc22a18* (Dao *et al.* 1998), *Slc22a2* and *Slc22a3* (Zwart *et al.* 2001), and the paternally expressed *Slc38a4* (Smith *et al.* 2003).

Some imprinted genes are expressed in both embryo and placenta, with the reciprocally imprinted *Igf2* and *Igf2r* genes representing classic examples. The paternally expressed *Igf2* is essential for embryonic and placental development, with paternal inheritance of an *Igf2* null allele resulting in small placenta and growth-retarded offspring, which are viable and remain small into adulthood (Baker *et al.* 1993; DeChiara *et al.* 1990). The maternally expressed *Igf2r* encodes a signalling antagonist to Igf2; maternal inheritance of a mutant

Igf2r allele causes fetal overgrowth and early post-natal lethality due to cardiac defects (Lau *et al.* 1994).

Further to the finding that imprinted genes affect neonatal growth; a recently emerging field is the discovery that some imprinted genes affect the behaviour of the mother and/or offspring in the neo-natal period. For instance mutation of the paternally expressed gene *Peg3* adversely affects suckling in pups and the maternal care exhibited by these animals in adulthood (Curley *et al.* 2004). The maternally expressed imprinted gene *Nesp* has also been implicated in affecting behavioural traits. *Nesp* deficient mice display increased exploratory behaviour when exposed to a novel environment, although this effect is diminished following acclimatisation of the animal to the environment (Plagge *et al.* 2005).

Imprinted genes in humans perform similar functions in regulating embryonic and placental development, with aberrant expression or mutation of imprinted genes associated with a variety of growth abnormalities, including Beckwith-Wiedemann Syndrome (BWS) (Hatada *et al.* 1996), PWS/AS (Jay *et al.* 1997; Knoll *et al.* 1989; Nicholls *et al.* 1989; Ozcelik *et al.* 1992), Silver-Russell Syndrome (Yoshihashi *et al.* 2000) and cancer (Rainier *et al.* 1993). Mouse models have been generated for a number of these diseases by targeting the gene(s) concerned, giving rise to similar phenotypes to the human conditions, and supporting a role for imprinted genes in human disease. For instance, many of the characteristics of human BWS patients are recapitulated in a mouse model possessing a *Cdkn1c* null allele in addition to LOI of *Igf2* (Caspary *et al.* 1999).

1.5 The evolution of imprinting

In the context of this investigation, genomic imprinting refers to the parent-of-origin specific monoallelic expression of specific autosomal genes. However, the term has historically been used in reference to similar regulatory mechanisms in a variety of species. Genomic imprinting was first used to describe the parent-of-origin specific elimination of sex chromosomes in the insect species *Sciara* (Crouse 1960). The term was subsequently used to describe the epigenetic silencing of the entire paternal genome in male mealybugs, thus apparently fulfilling a role in sex determination (Brown and Nur 1964). Parent-of-origin dependent phenotypes have also been observed in plants; for instance, differing

pigmentation patterns in maize depend upon the parental inheritance of a particular allele (Kermicle 1970). Imprinted genes are also expressed during endosperm development in some flowering plant species (Kinoshita *et al.* 1999; Vinkenoog *et al.* 2003). In animals, imprinted genes are observed in placental mammals (eutheria and marsupials), but not in monotremes or invertebrate species (John and Surani 2000). Interestingly, in addition to the imprinting of autosomal genes, the X chromosome is imprinted in the extraembryonic tissue of mice with the paternally inherited X chromosome preferentially inactivated (Takagi and Sasaki 1975; West *et al.* 1977).

The evolution of imprinting has occurred independently in plants and animals, although similar mechanisms of gene regulation are observed, thus representing a case of convergent evolution (Feil and Berger 2007). The selection of monoallelic expression over biallelic expression, and the parental-specific allele expression of imprinted genes has generated much debate, and the development of a number of theories attempting to explain the evolution of imprinting in mammals (reviewed in Hurst 1997; Hurst and McVean 1998), some of which are discussed below.

1.5.1 Ovarian time-bomb hypothesis

Imprinted genes represent the primary preventative factor of parthenogenetic development in mammals (Kono 2006). The "ovarian time-bomb hypothesis" purports that this was the driving force of imprinting, suggesting that imprinting arose as a mechanism to prevent unfertilised oocytes from implanting and developing into malignant trophoblast disease (Varmuza and Mann 1994).

Benign ovarian tumours resulting from parthenogenetically activated oocytes occur in around 1-2% of women, whereas germ-cell derived testicular cancer in males occurs at a rate approximately 1000-fold lower (Varmuza and Mann 1994). Trophoblast is an inherently invasive tissue type, and this level of spontaneous tumour development could have potentially serious consequences for the reproductive success and survival of an individual. The authors proposed that ovarian tumours are prevented from becoming invasive by imprinting and silencing of specific genes that promote trophoblast proliferation and invasion (Varmuza and Mann 1994). Consequently, maternally expressed imprinted genes would tend to restrict extraembryonic tissue growth, with genes promoting growth

and invasion of the placenta expressed only from the paternal genome. Support for this theory is provided by phenotypic characterisation of complete hydatidiform molar pregnancies. Such conceptuses result from fertilisation of an enucleated egg and duplication of the paternal genome, and thus are entirely androgenetic in origin. The result is the development of a large mass of potentially highly invasive extraembryonic tissue (Kajii and Ohama 1977). In contrast, partial hydatidiform moles, which contain an additional maternal genetic contribution, exhibit some embryonic development with generally less invasive extraembryonic tissue (Devriendt 2005; Lawler *et al.* 1982). Furthermore, parthenogenetic reproduction and genomic imprinting are phylogentically mutually exclusive, as discussed in Section 1.2. The ovarian time-bomb hypothesis does not offer an explanation for either the presence of imprinting in the male genome, or the imprinting of genes that are not involved in trophoblast development, suggesting instead that such genes are "bystanders" of the imprinting mechanism (Varmuza and Mann 1994), and has been largely rejected as a consequence (Haig 1994; Moore 1994; Solter 1994).

1.5.2 Adaptation of host defence

Approximately half of the human genome is thought to have arisen from the insertion of transposable elements, the majority of which are no longer active (Lander *et al.* 2001). Integration of such transposable elements poses significant risk to the host organism, occasionally through the effect of integration into endogenous genes, but primarily due to the effect of the translated gene-product (reviewed in Yoder *et al.* 1997). The mammalian genome protects itself by inactivating these transposable elements by methylation and chromatin modifications (Birchler *et al.* 2000; Yoder *et al.* 1997) - the same mechanisms that control expression of imprinted genes (Li *et al.* 1993). The common usage of methylation and chromatin remodelling in these two processes led some to predict that imprinting of some genes evolved as a consequence of these host defence mechanisms (Barlow 1993; Ono *et al.* 2001). Although some imprinted genes exhibit retrotransposon-like features, for instance *Peg10* (Ono *et al.* 2001), many other imprinted genes bear no resemblance to transposable DNA sequences, although the presence of such sequences within non-coding regions of imprinted domains cannot be excluded.

1.6.4 Placentation hypothesis

Mammals are divided into three broad groups: monotremes, marsupials and eutheria. Monotremes are egg-laying mammals, whereas marsupials and eutheria (collectively referred to as theria) are viviparous, giving birth to live, well-developed young. Therians diverged from monotreme ancestors approximately 170 million years ago, whereas eutheria diverged from marsupials around 150 million years ago (Figure 1.4) (Bininda-Emonds *et al.* 2007). The viviparous reproduction exhibited by the two groups of therian mammals is possible due to the appearance of the placenta around the time of divergence from monotremes. Although they share a common placental ancestor, the eutherian and marsupial placentae subsequently evolved independently. Marsupials have a relatively non-invasive placenta, which supports only short gestation periods, with gestation completed in the pouch. In contrast, eutherian mammals generally possess more advanced and invasive placental structures, and thus can support longer gestation periods, allowing the birth of more advanced young (reviewed in John and Surani 2000).

Genomic imprinting emerged in the same evolutionary time-period as placentation. Over 100 imprinted genes have been identified in eutheria, the majority of which are expressed in the placenta. A subset of which are also imprinted in marsupials, although the imprinting status is not conserved for all genes between marsupials and eutheria. For example, although the paternal expression of *Peg1* is conserved in marsupials, *Cdkn1c* does not exhibit parental-specific expression in this order (Suzuki *et al.* 2005). Imprinting has thus far not been described in monotremes or other vertebrates such as birds or reptiles, with the reciprocally imprinted *Igf2* and *Igf2r* exhibiting biallelic expression in chickens and monotremes, whereas *Igf2* is paternally expressed and *Igf2r* is maternally expressed in eutheria and marsupials (Killian *et al.* 2000; Nolan *et al.* 2001; Suzuki *et al.* 2005).

The "placenta hypothesis" suggests that imprinting of specific genes was driven by the evolution of the placenta, which exerted selective pressure on genes that regulate embryonic growth and development (Hall 1990; Kaneko-Ishino *et al.* 2003). Following the divergence of marsupials and eutheria, co-evolution of the placenta and imprinting resulted in the distinct placental

structures and reproductive strategies of the two groups. This hypothesis accounts for the phylogenetic distribution of imprinting and the role of such genes in regulating embryonic growth and development.

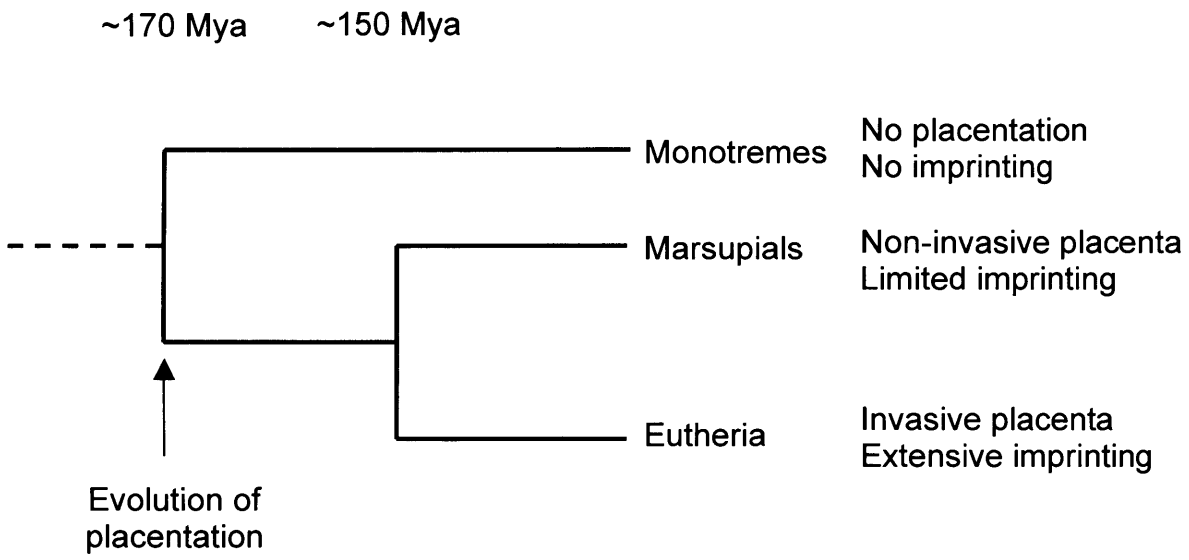


Figure 1.4: Co-evolution of placentae and imprinting

Marsupials and eutherian mammals shared a common placental ancestor around 170 Mya. Placentation co-evolved with genomic imprinting, with imprinting of specific genes enabling the subsequent evolution and increased invasiveness of the eutherian placenta following divergence from marsupials around 150 Mya. Mya = Million years ago.

1.5.3 Parental conflict hypothesis

Perhaps the most widely debated theory regarding genomic imprinting in mammals is the "parental conflict hypothesis", first proposed by Moore and Haig (1991). The conflict hypothesis predicts that imprinting arose in placental mammals due to the "*conflicting interests of maternal and paternal genes in relation to the transfer of nutrients from the mother to her offspring*". Both parents are invested in producing large, viable offspring that will successfully reproduce, but the mother must additionally preserve resources for herself and future progeny. This hypothesis suggests that paternally expressed imprinted genes tend to be growth promoting, whereas maternally expressed imprinted genes tend to be growth restrictive (Moore and Haig 1991). The reciprocally imprinted *Igf2/Igf2r* genes provided the first evidence in support of the parental conflict hypothesis. The growth promoting *Igf2* gene is paternally expressed whereas the

growth restrictive *Igf2r*, which encodes a signalling antagonist of Igf2, is maternally expressed (Barlow *et al.* 1991; DeChiara *et al.* 1991; Haig and Graham 1991). Indeed, expression patterns for the majority of imprinted genes for which a function has been assigned correlate with these predictions (Isles and Holland 2005; Tycko and Morison 2002).

The importance of imprinting in the placenta is further support for the parental conflict hypothesis (Tycko 2006). The placenta mediates the maternal supply and fetal demand for nutrients during gestation, and thus directly influences embryonic growth (Reik *et al.* 2003). According to the conflict hypothesis, paternally expressed imprinted genes would be expected to enhance placental function, whereas maternally expressed imprinted genes would be expected to restrict placental capacity. This is true for *Igf2*, with loss of expression of the paternal transcript in the placenta causing reduced placental size; conversely, loss of the maternally expressed *Cdkn1c* causes placentomegaly (Constância *et al.* 2002; DeChiara *et al.* 1990; DeChiara *et al.* 1991; Takahashi *et al.* 2000a).

In addition to explaining the common functions of imprinted genes, the conflict hypothesis also attempts to explain the phylogenetic distribution of imprinting to placental mammals. Monoallelic expression of specific genes in the placenta achieved by imprinting effectively restricts the effects of these gene products, thus providing an optimal environment for nutrient delivery to the embryo whilst maintaining sufficient resources for maternal health. Vertebrate species such as reptiles and birds, which reproduce by laying eggs, are not subject to the same continued nutritional demands as those experienced by placental mammals, and thus would not be expected to exhibit imprinting.

The conflict hypothesis is not however without its limitations (reviewed in Hurst and McVean 1998); for instance the imprint status of some genes does not correlate with the pattern predicted by the hypothesis. Most notable of these is the maternally expressed *Ascl2* gene. *Ascl2* null mice have small placenta, with a complete loss of the spongiotrophoblast region, causing embryonic lethality due to placental failure (Guillemot *et al.* 1994). According to the conflict hypothesis, maternally expressed genes should be growth-restrictive and thus loss of expression would be predicted to result in some form of placentomegaly, whereas over-expression would restrict placental growth. However, loss of

expression does not model imprinting since it tests the function of the gene and not the consequences of altered expression. It will be of particular interest to determine whether over-expression of *Ascl2* in the mouse placenta has a consequence.

1.6 The placenta

The placenta is a transient organ that is essential for mammalian embryogenesis, developing early in pregnancy before being discarded at birth. Comprised largely of extraembryonic trophoblast cells, the placenta is permeated by embryo-derived blood vessels from the umbilical cord and maternal blood vessels from the uterus. Growth, development and ultimate survival of the embryo depends upon this organ, as exemplified by many mouse models of disrupted placentation, in which embryonic growth is impaired and often associated with embryonic lethality (reviewed in Cross *et al.* 2003a; and Rossant and Cross 2001).

The primary role of the placenta is to provide sufficient nutrients for fetal growth, achieved through the formation of intimate feto-maternal interfaces. The functions of the placenta can be broadly categorised as "transport and barrier" and "invasive and endocrine" functions (Cross *et al.* 2003a; Georgiades *et al.* 2002). Transport and barrier functions include the supply of nutrients to and the removal of waste products from the embryo, and the protection of the embryo from potential maternal immune response (Georgiades *et al.* 2002; Koch and Platt 2007). Invasive and endocrine functions include the invasion of placental cells into the uterus wall to anchor the placenta in place, and the secretion of hormones into maternal blood that alter maternal metabolism to favour embryonic growth and increase blood flow to the placenta (Cross *et al.* 2003a; Malassine *et al.* 2003).

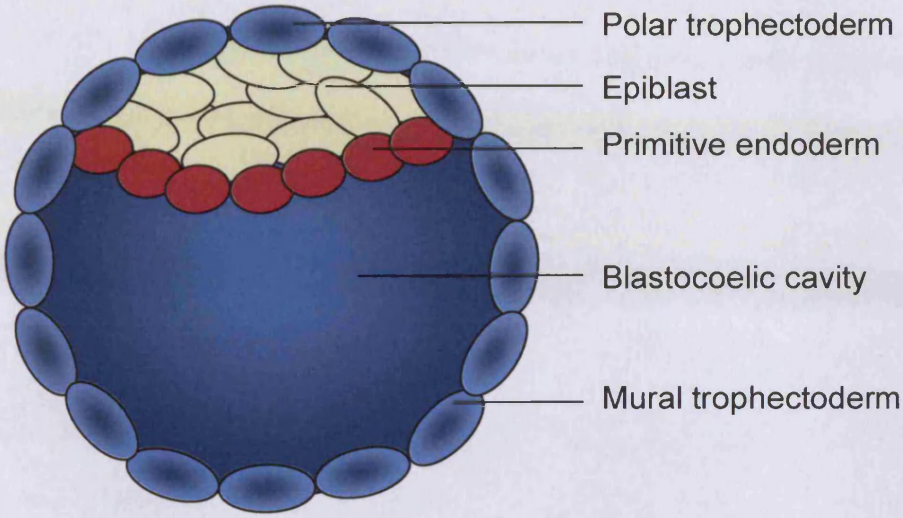
The vital role that the placenta plays in nourishing the embryo means that placental defects may have serious consequences for embryonic growth. In humans, placental defects are associated with pregnancy complications including pre-eclampsia and intrauterine growth restriction (IUGR). Mouse models provide useful tools for gaining further understanding of such conditions and for understanding placental development in general.

1.6.1 Development of the mouse placenta

The trophoblast cell lineage is the first to differentiate during embryogenesis, occurring at around embryonic day 3 (E3.0) (Gilbert 2003; Pedersen 1986). At this stage the zygote has proliferated to form a ball of approximately 16 cells (the morula), comprising a monolayer of trophoblast cells, called the trophectoderm, surrounding the inner cell mass (ICM) (Gilbert 2003). The ICM forms the entire embryo, with the trophectoderm contributing solely to the extraembryonic tissue (Cross 2005; Rossant and Cross 2001; Watson and Cross 2005). Cell fate can be experimentally manipulated at this stage by altering cell position, indicating that cells of the morula remain totipotent (Ziomek *et al.* 1982). As embryogenesis progresses to the 32-cell blastocyst stage, the ICM is characterised by expression of the transcription factors *Oct4* and *Nanog*. Expression of these genes is repressed by Cdx2, which is specifically expressed by cells of the trophectoderm (Hart *et al.* 2004; Kunath *et al.* 2004; Palmieri *et al.* 1994; Strumpf *et al.* 2005). Deletion of *Cdx2* causes implantation failure due to loss of the extraembryonic cell lineage (Strumpf *et al.* 2005).

Prior to implantation, water enters the blastocyst under osmotic flow forming the blastocoelic cavity, to one side of which lies the ICM (Fig 1.5A) (Gilbert 2003; Pedersen 1986). Trophectoderm cells lying directly above the ICM are referred to as polar trophectoderm, whereas those cells not in contact with the ICM are designated mural trophectoderm (Pedersen 1986). The ICM comprises a heterogeneous population of epiblast and primitive endoderm cell lineages (Chazaud *et al.* 2006). Epiblast cells segregate closest to the trophoblast cells and contain precursors to all embryonic cell types. Epiblast cells are surrounded by a monolayer of primitive endoderm cells, called the visceral endoderm. These primitive endoderm cells spread across the inner surface of the blastocoelic cavity, beneath the primary trophoblast cell layer forming the parietal endoderm (Lawson and Pedersen 1987). Together, the parietal endoderm and giant cell layer form the parietal yolk sac, while the visceral endoderm combines with the embryonic-derived extraembryonic mesoderm to form the visceral yolk sac (Jollie 1990). The parietal and visceral yolk sacs facilitate nutrient uptake to the embryo, prior to the establishment of the mature placenta.

A E3.5 blastocyst



B E6.5 egg cylinder stage

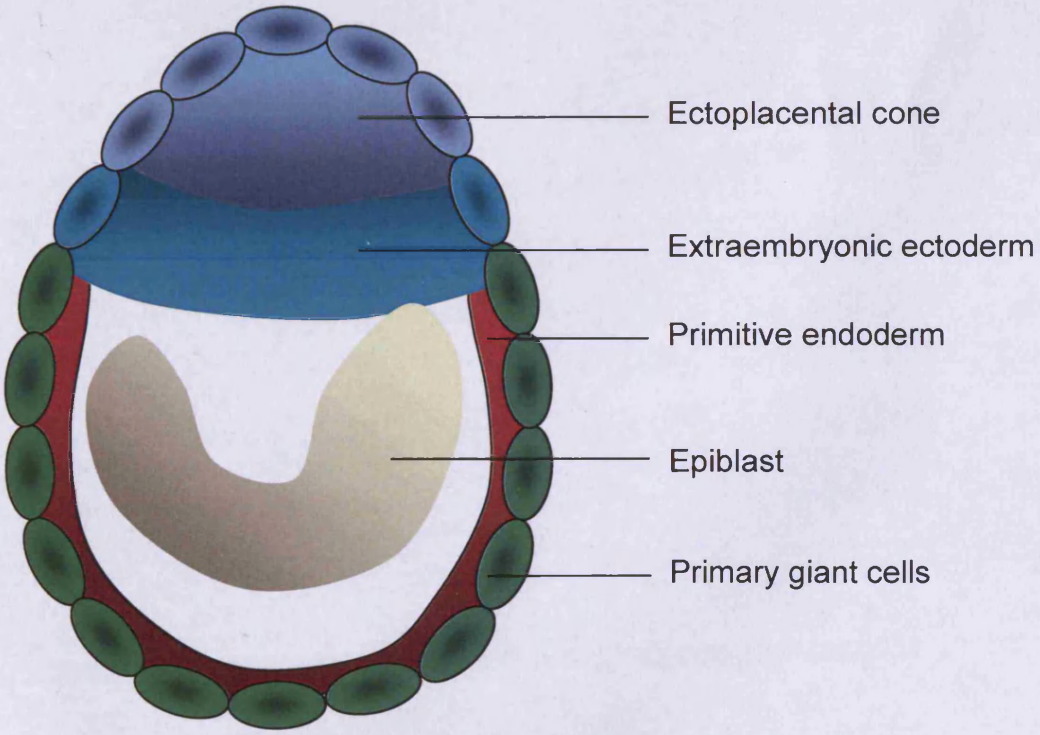


Figure 1.5: Early differentiation of trophoblast lineages

A: At E3.5 the blastocyst comprises a monolayer of trophectoderm cells surrounding a blastocoelic cavity. The ICM is attached to one side of the trophectoderm and consists of cells of the epiblast and primitive endoderm lineages. B: Polar trophectoderm cells directly above the ICM proliferate outwards to form the ectoplacental cone and inwards to form the extraembryonic ectoderm. The remaining mural trophectoderm undergo endoreduplication to form primary giant cells, which together with the primitive endoderm forms the parietal yolk sac.

Following implantation of the blastocyst into the uterine wall at E4.5, polar trophectoderm cells proliferate inwards to form the extraembryonic ectoderm and divide outwards to form the ectoplacental cone (Copp 1979). In contrast, mural trophectoderm cells cease proliferating, instead undergoing repeated rounds of DNA replication resulting in the formation of large, polyploid primary trophoblast giant cells through the process of endoreduplication (Rossant and Cross 2001; Watson and Cross 2005). This stage of embryogenesis is referred to the egg-cylinder stage, and corresponds to approximately E6.5 (Fig 1.5B). Continued proliferation of polar trophectoderm cells is maintained by the release of the growth factor Fgf4 from the ICM, acting through Fgf2r receptors of trophectoderm cells (Haffner-Krausz *et al.* 1999; Niswander and Martin 1992). Consequently, *Fgf4* and *Fgf2r* mutant mice exhibit similar phenotypes, dying soon after implantation due to a failure of trophectoderm development (Arman *et al.* 1998; Feldman *et al.* 1995). Fgf4 signals are transmitted downstream by Erk2/Mapk1, mutation of which also results in a failure of ectoplacental cone and extraembryonic ectoderm formation (Saba-El-Leil *et al.* 2003).

Expansion and differentiation of the ectoplacental cone forms a compact layer of cells called the spongiotrophoblast. Similar to mural trophectoderm cells, cells at the maternal edge the spongiotrophoblast undergo endoreduplication becoming secondary trophoblast giant cells (Cross 2000; Cross *et al.* 1994). Meanwhile, the extraembryonic ectoderm proliferates to generate the chorionic epithelium, initially forming a flat "chorionic plate" at the base of the placenta. Simultaneously the allantois (umbilical cord) develops from the posterior end of the embryo (Rossant and Cross 2001; Watson and Cross 2005). At E8.5, the allantois and chorionic epithelium fuse in an event termed chorioallantoic attachment (Downs and Gardner 1995; Watson and Cross 2005). Following chorioallantoic attachment, the chorionic epithelium begins to undergo branching morphogenesis, forming primary villi at regularly spaced intervals. Fetal blood vessels from the allantois begin to grow into the primary villi, which elongate and branch further to form the characteristic maze-like structure of the murine labyrinth (Cross *et al.* 2003b).

Simultaneous with branching morphogenesis of the chorionic epithelium, trophoblast cells of the extraembryonic ectoderm differentiate to form the trilaminar arrangement of the labyrinth layer (Fig 1.6). In addition to the epithelial

lining of fetal blood vessels, the maternal and fetal blood flows are separated by three layers of trophoblast cells. A single layer of mononuclear sinusoidal trophoblast giant cells (S-TGCs) replaces the endothelial lining of maternal vasculature. A bilayer of multinucleated syncytiotrophoblast formed by cell fusion surrounds the fetal vasculature, which maintains its endothelial cell lining, unlike maternal vessels (Coan *et al.* 2005b; Simmons and Cross 2005; Simmons *et al.* 2008). The S-TGCs primarily perform a secretory function, releasing hormones into maternal blood, whereas the syncytiotrophoblast layers are involved in nutrient transport (Georgiades *et al.* 2002; Simmons and Cross 2005; Watson and Cross 2005).

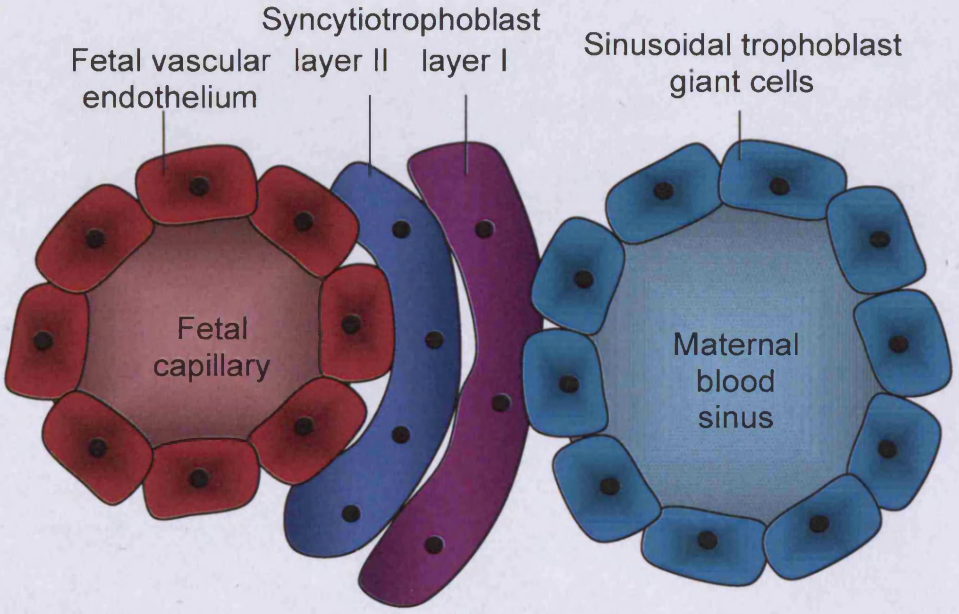


Figure 1.6: Trilaminar arrangement of labyrinth

The fetal and maternal blood flows are separated by a trilaminar arrangement of trophoblast cells in the labyrinth layer of the mouse placenta. The endothelial lining of maternal vasculature is replaced by sinusoidal trophoblast giant cells. A bilayer of syncytiotrophoblast cells formed by cell fusion separates maternal blood sinuses from fetal capillaries. Fetal capillaries maintain their endothelial lining, thus separating maternal and fetal blood by four cell layers.

Mouse models of defective placentation indicate that of the genes examined that are involved in placental development, the majority appear to control development of the labyrinth region (see Tables 2 and 3 in Watson and Cross 2005). Perhaps one of the most crucial genes required for development of the labyrinth region is *Gcm1*. Expression is first detected at around E7.5 in distinct clusters of cells in the extraembryonic ectoderm, and regulates the process of branching morphogenesis in the labyrinth region, with expression restricted to cells at the tip of branching villi (Basyuk *et al.* 1999). Loss of *Gcm1* expression results in failure of branching morphogenesis with the chorionic plate remaining flat and undifferentiated. As a consequence of this placental failure, *Gcm1*-null embryos die at ~E10.5 (Anson-Cartwright *et al.* 2000).

The definitive mouse placenta is formed by ~E12.5 and comprises three layers, namely the labyrinth, spongiotrophoblast (junctional zone) and outer monolayer of giant cells in addition to the maternal decidua into which the placenta embeds (Fig 1.7). A sub-population of spongiotrophoblast cells called glycogen cells begin to accumulate their characteristic glycogen stores at this stage. Glycogen cells are characterised by a large, glycogen-filled cytoplasm and expression of the spongiotrophoblast-specific marker *Tpbpa* (*4311*), suggesting that they are a direct sub-type of the spongiotrophoblast (Adamson *et al.* 2002). However, the actual origin of these cells remains elusive, with expression of the glycogen cell-specific marker *protocadherin 12* (*Pcdh12*) detected in the ectoplacental cone as early as E7.5 (Bouillot *et al.* 2005), prior to the differentiation of spongiotrophoblast and appearance of the morphological characteristics of glycogen cells. Furthermore, the placentae of *Cdkn1c*-null mice possess approximately twice the normal number of spongiotrophoblast cells, whereas the number of glycogen cells remains similar to wild-type (Takahashi *et al.* 2000a). Between E14.5 and term, glycogen cells migrate into the decidua where they tend to cluster around maternal blood vessels. In addition to the large glycogen stores, these cells are also characterised by synthesis of the growth factor Igf2. Interestingly, both glycogen cell number and glycogen content are greatly reduced in *Igf2*-null placenta, indicating that glycogen cell differentiation and glycogen synthesis/uptake are *Igf2*-dependent processes (Lopez *et al.* 1996). The function of glycogen cells remains unclear, although a role in parturition has been proposed (Coan *et al.* 2006).

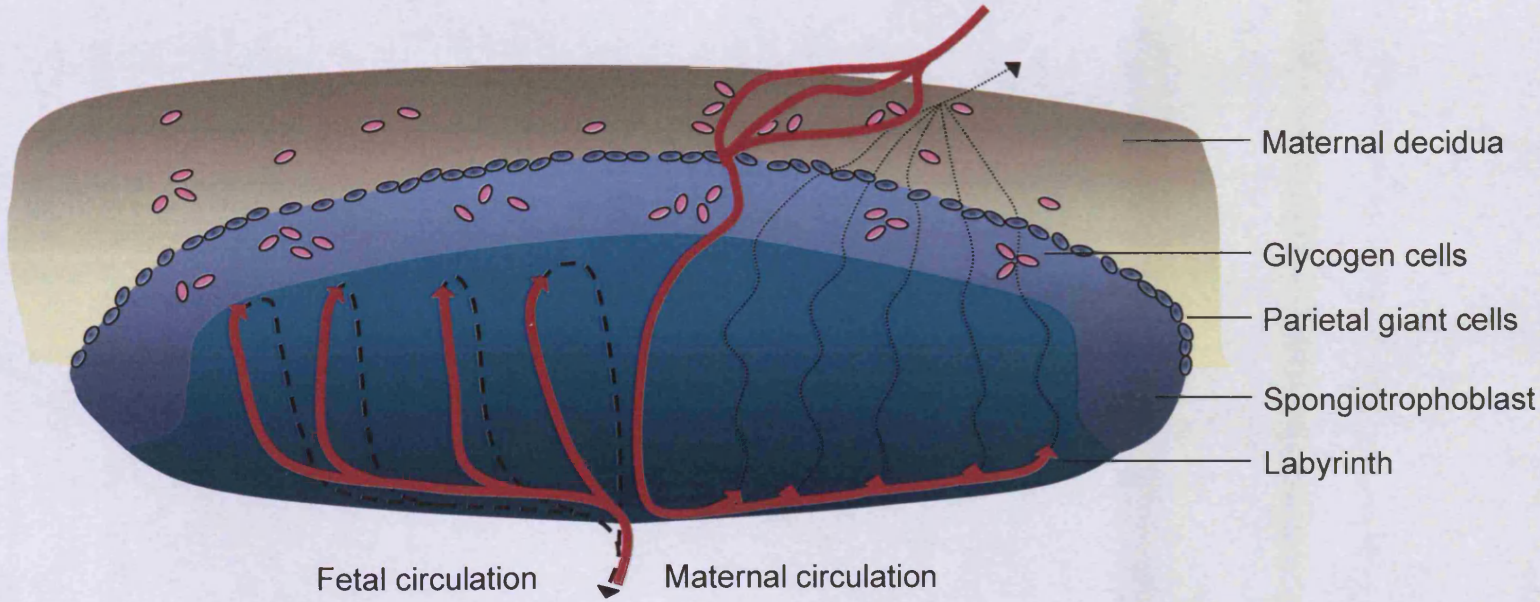


Figure 1.7: Structure and blood circulation of the mature mouse placenta

The mature mouse placenta comprises three main regions; the labyrinth, spongiotrophoblast and maternal decidua. Separating the spongiotrophoblast from the maternal decidua is a discontinuous layer of invasive trophoblast giant cells. Glycogen cells first arise at E12.5 in the spongiotrophoblast and migrate to the decidua until term. Fetal and maternal blood flow in a counter-current direction. Maternal blood is delivered to the base of the placenta through arterial canals and drains back to the maternal side through blood sinuses. Fetal blood enters at the base of the placenta, traverses the labyrinth in small arterioles and returns back to the embryo through capillaries.

In addition to the S-TGC subtype described previously, a further three sub-types of trophoblast giant cell (TGC) have been described in the mouse placenta. The secondary giant cell layer separating the spongiotrophoblast and maternal decidua is comprised of parietal giant cells (P-TGCs). Spiral artery-associated trophoblast giant cells (SpA-TGCs) surround the maternal spiral arteries in the decidua, whereas canal-associated trophoblast giant cells (C-TGCs) surround the maternal blood canals that traverse the spongiotrophoblast and enter the labyrinth layer of the placenta (summarised in Figure 1.8). All giant cell subtypes are characterised by a large, polyploid nucleus and are identified by specific gene expression markers and their spatial localisation in the placenta (reviewed in Hemberger 2008).

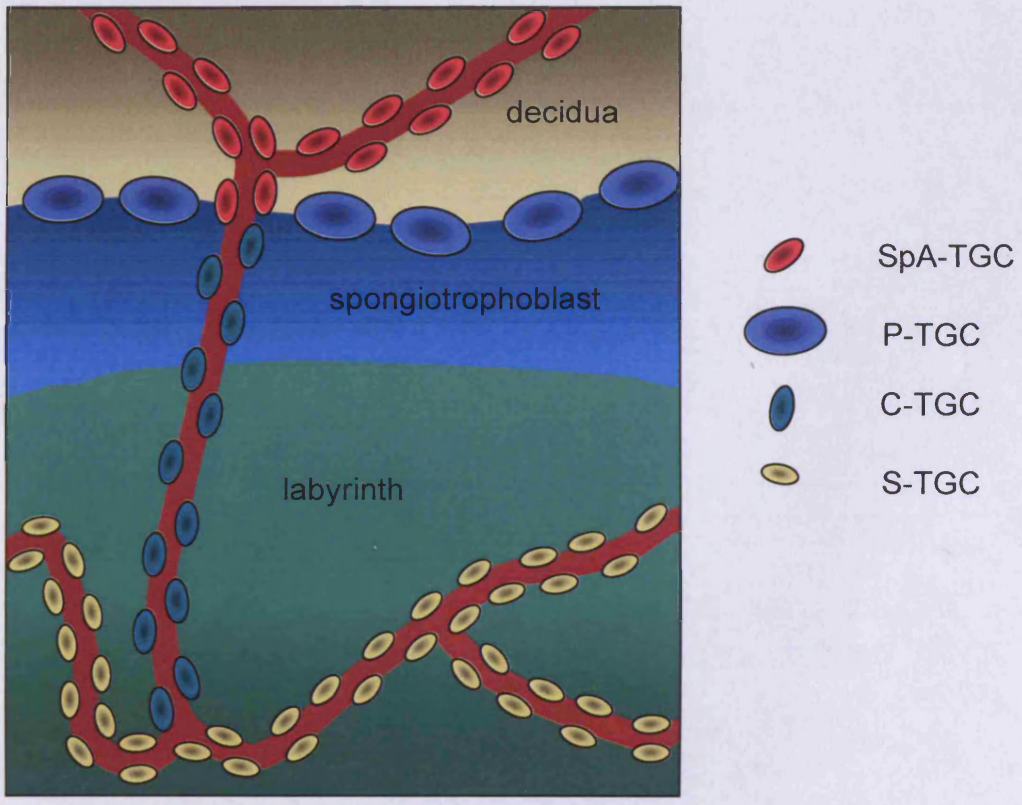


Figure 1.8: Trophoblast giant cell subtypes in the mouse placenta

Four sub-types of trophoblast giant cell exist in the mouse placenta. The parietal trophoblast giant cells (P-TGCs) form the boundary between the maternal decidua and spongiotrophoblast. Spiral arteries entering the decidua are surrounded by spiral artery associated trophoblast giant cells (SpA-TGCs), whereas canal-associated trophoblast giant cells (C-TGCs) surround the blood canals that traverse the spongiotrophoblast and deliver maternal blood to the labyrinth. The endothelial lining of maternal vasculature in the labyrinth is eroded and replaced by sinusoidal trophoblast giant cells (S-TGCs). Diagram adapted from Hemberger (2008).

Prior to the completion of placental development ~E12.5 the developing fetus is nourished by the uptake of nutrients across the yolk sac (Cross *et al.* 1994; Georgiades *et al.* 2002). Between E10.5 and E12.5, the maternal and fetal blood supplies to the placenta are complete, with the fetus subsequently obtaining all required nutrients by transfer across the placenta. This is a critical stage in placental development, as exemplified by the significant number of mouse models of defective placentation that are embryonic lethal at this stage, including mice deficient for *Ascl2* (Guillemot *et al.* 1994), *Gcm1* (Anson-Cartwright *et al.* 2000), and *Vhl* in which the fetal placental vasculature fails to form correctly (Gnarra *et al.* 1997).

Effective placental function requires that maternal and fetal blood circulations be brought into close proximity such that exchange of nutrients, wastes and gases can take place. Maternal blood enters the placenta from the uterine radial artery, which splits into numerous spiral arteries in the decidua, before converging at the giant cell layer to form between one and four arterial canals (Adamson *et al.* 2002). The endothelial lining of these central canals, which cross the spongiotrophoblast layer, is eroded away and replaced with trophoblast giant cells (Rossant and Cross 2001). Maternal blood is carried directly to the base of the placenta where it flows into the trophoblast-lined maternal blood sinuses, and subsequently drains back to the decidua (Adamson *et al.* 2002). Fetal blood enters at the base of the placenta through a single artery, subsequently branching into a number of smaller arterioles, which penetrate the labyrinth and upon reaching the spongiotrophoblast, form capillaries that direct fetal blood out of the placenta (Adamson *et al.* 2002). Maternal and fetal blood thus flows in a counter-current direction, with maternal blood draining towards the decidua through trophoblast-lined maternal sinuses as fetal blood is carried back to the fetus through endothelial-lined capillaries.

1.6.2 Comparison of human and mouse placenta

Practical and ethical considerations prevent the direct investigation of human placental development, and thus a suitable model organism must be used in which to undertake comparative studies. The mouse is a well-established model organism in numerous fields, and provides an ideal model in which to study the placenta. A key advantage of the mouse is the relatively short gestation time, in

addition to the ability to give birth to multiple pups in the same litter. This enables a direct comparison of wild type and mutant individuals in the same litter. In order to relate findings from mouse models to the human placenta, it is important to understand the similarities and differences between placenta of the two organisms, and thus the potential limitations of mouse studies.

Human and mouse placentae are described as discoid, as they are generally dome shaped with a flat surface facing the embryo to which the allantois attaches (Georgiades *et al.* 2002). Both human and mouse placentae comprise three distinct layers. The labyrinth region of the mouse placenta is analogous to the "fetal placenta" in humans. In the human placenta, a single layer of syncytiotrophoblast cells separates maternal blood from fetal capillaries, in comparison to the trilaminar arrangement in the mouse. Both human "monochorial" and mouse "trichorial" placentae possess "haemochorial interfaces", with fetal capillaries bathed directly in maternal blood (Georgiades *et al.* 2002). Differences exist in the branching of fetal vasculature between the two species, with the human "villous" structure resembling a tree, with progressive branching and sub-branching, whereas the branches of the murine vasculature are interconnected to form a complex "labyrinth" arrangement. Maternal blood sinuses in the human placenta are thus relatively larger than their counterparts in the mouse (Georgiades *et al.* 2002; Kingdom *et al.* 2000).

Lying directly above the fetal placenta/labyrinth is a compact region of cytotrophoblast cells called the basal plate in humans and the spongiotrophoblast in mice. Glycogen cells are observed in this layer in both humans and mice, and although this region does not contain fetal vasculature, trophoblast-lined maternal arterial and venous channels dissect this zone. The function of this region is unclear, although it is suggested to fulfil an endocrine/secretory role in both species. A third layer, consisting of polyploid secondary giant cells, surrounds the spongiotrophoblast in mice and invades the uterine wall. The extravillous cytotrophoblast cells in humans, which are polyploid although to a lesser extent, appear to perform a similar invasive role (Georgiades *et al.* 2002; Rossant and Cross 2001). The primary differences between mouse and human placental structure are summarised in Tables 1.2 and 1.3.

Mouse	Human
Labyrinth	Fetal placenta
Maternal blood space/lacunae	Intervillous space
Spongiotrophoblast/Junctional zone *comprised of:* spongiotrophoblast cells glycogen cells	Basal plate *comprised of:* cytotrophoblast cell columns glycogen cells
Giant cells	Giant cells*
Decidua	Placental bed

Table 1.2: Comparison of human and mouse placental structure

The above table compares the nomenclature for the major regions of the mouse and human placenta. *Although giant cells are observed in human placenta, they are morphologically distinct. An analogous cell type to the murine giant cells thus appears to be the invasive extravillous trophoblast.

Structure	Mouse	Human
Chorionic plate	Maze-like and interconnected Small maternal blood spaces	Tree-like with branches and sub-branches Larger intervillous space
Ultrastructure	Trichorial: Three trophoblast layers separate fetal and maternal blood	Monochorial: A single trophoblast layer separate fetal and maternal blood

Table 1.3: Comparison of structure of labyrinth/fetal placenta

The table summarises the two major differences between the organisation of the labyrinth layer of the mouse placenta and the analogous fetal placental layer of the human placenta.

1.6.3 Imprinted gene expression in the mouse placenta

The majority of imprinted genes are expressed in the placenta (reviewed in Coan *et al.* 2005a). This pattern of expression is consistent with their role in regulating fetal growth by controlling mediating the supply of nutrients to the embryo during gestation. Imprinted genes are involved in both the control of placental development (*Ascl2*, *Peg10*, *Grb10*) and the transport of nutrients (*Slc22a2*, *Slc22a3* and *Slc38a4*). Although placental expression has been reported for the majority of imprinted genes, the spatial expression pattern has been reported for only a small number of these genes. Table 1.4 summarises the expression pattern of the most extensively studied of these genes.

Gene	Spatial expression
Ascl2	Diploid trophoblast of labyrinth and spongiotrophoblast layers until E12.5, declining thereafter
Cdkn1c	Labyrinth, spongiotrophoblast, glycogen cells, giant cells
Dlk1	Fetal endothelium of labyrinth region
Igf2	Labyrinth, spongiotrophoblast, glycogen cells, giant cells and fetal endothelium
*Igf2*P0	Labyrinth
Mest/Peg1	Chorionic plate and fetal endothelium (not expressed in trophoblast cells)
Peg3	Spongiotrophoblast, giant cells and weak expression in labyrinth
Phlda2	Ectoplacental cone (at ~E5.5), thereafter restricted to labyrinth

Table 1.4: Summary of imprinted gene expression in the mouse placenta

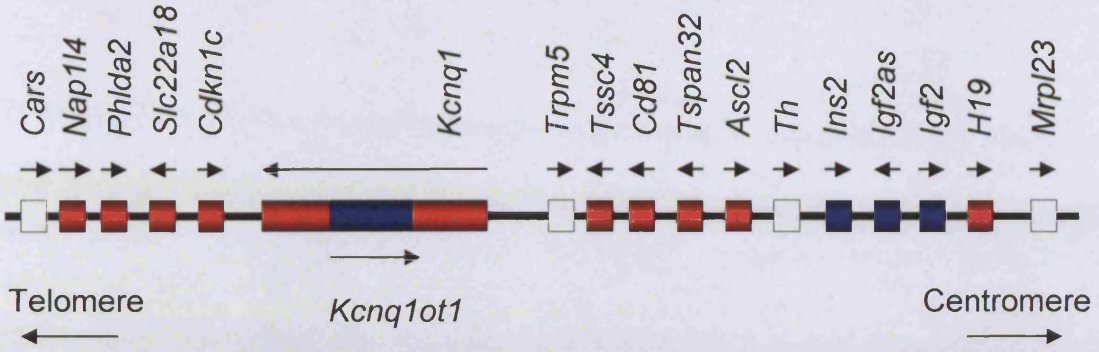
The table summarises the reported spatial expression pattern of autosomal imprinted genes in the mouse placenta. For many imprinted genes, although placental expression has been reported at high levels, the spatial expression pattern has not yet been described. Table adapted from Coan *et al.* (2005a) and references therein. Red = maternally expressed; Blue = paternally expressed.

1.7 Mouse distal 7 and human 11p15.5

A major imprinted domain implicated in placental development is located on mouse distal chromosome 7, a region that is homologous to the human BWS-associated cluster at 11p15.5. Mouse distal 7 is approximately 1 megabase (Mb) in length and contains thirteen protein-coding genes, eleven of which exhibit imprinted expression to varying degrees. The domain also contains three imprinted genes from which non-coding antisense RNA is transcribed (reviewed in Ainscough *et al.* 1998). In comparison, the human region is 40% longer, orientated in the opposite direction with respect to the centromere and contains an additional two imprinted genes that encode antisense RNA transcripts (Cooper *et al.* 1998; Paulsen *et al.* 1998; Paulsen *et al.* 2000; Xin *et al.* 2000). The non-imprinted genes *Cars* and *Mrpl23* approximately demarcate the telomeric and centromeric extents of the mouse domain respectively (Engemann *et al.* 2000; Zubair *et al.* 1997). Due to the reverse orientation of the domain in human, *MRPL23* marks the telomeric extent, whereas *NAP1L4*, which is imprinted in mouse but not human, marks the centromeric extent of 11p15.5 (Hu *et al.* 1996; Tsang *et al.* 1995). Although the imprinting status is conserved for most genes in the domain, some are imprinted only in humans and others only in the mouse (summarised in Fig 1.9).

Two imprinted sub-domains have been described within mouse distal 7, each controlled by an independent regulatory element (Lee *et al.* 1999b). The IC1 domain is located at the centromeric end of distal 7, and incorporates the maternally expressed antisense *H19* and the paternally expressed *Ins2*, *Igf2* and its associated antisense transcript *Igf2as*. The IC2 domain encompasses the genes between *Ascl2* and *Nap1l4*, with the biallelically-expressed *Th* forming a boundary between the two sub-domains. Interestingly, *TSSC4*, *CD81* and *TSPAN32* are expressed biallelically in humans, but are maternally expressed in the mouse (Lee *et al.* 1999a; Monk *et al.* 2006). Furthermore, there is conflicting data as to whether *ASCL2* is imprinted (Alders *et al.* 1997) or not (Miyamoto *et al.* 2002; Monk *et al.* 2006; Westerman *et al.* 2001) in humans. In addition, *Trpm5*/*TRPM5*, which is not imprinted in the mouse, is preferentially expressed from the paternal allele in human (Paulsen *et al.* 1998; Prawitt *et al.* 2000).

A: Mouse Distal 7:



B: Human 11p15.5:

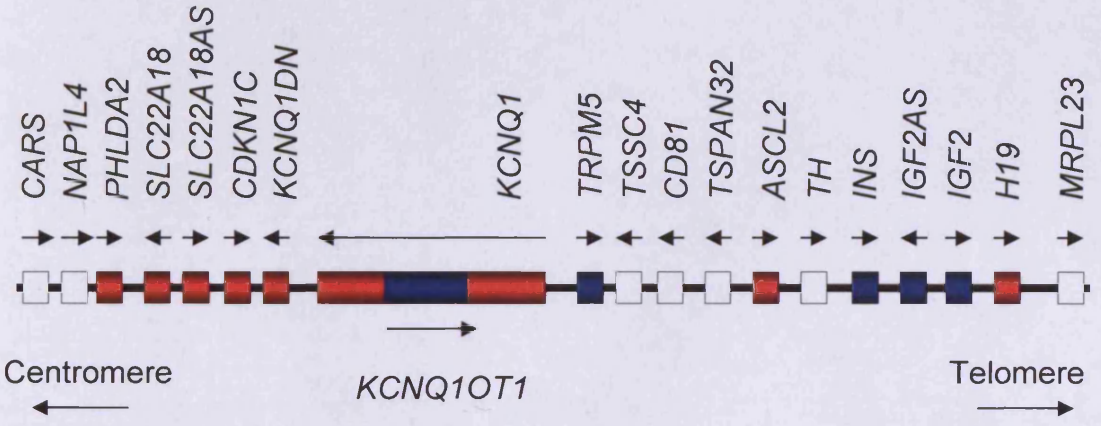


Figure 1.9: Domain structure of mouse distal 7 and human 11p15.5

The imprinting status of genes located within the syntenic mouse distal 7 (A) and human chromosome 11p15.5 (B) imprinted domains are depicted. In the mouse, the imprinted region consists of two sub-domains, IC1 (spanning *Ins2* to *H19*), and IC2, encompassing *Ascl2* to genes several hundred-kb upstream. The human and mouse domains share similar organisation and regulation, but are orientated in opposite directions, with the human domain around 40% longer. Red = maternally expressed genes; blue = paternally expressed genes; white = biallelically expressed genes. Direction of transcription is denoted by arrows. Diagram not to scale.

1.7.1 Control of imprinting at the mouse IC1 sub-domain

The IC1 sub-domain is one of the most extensively studied imprinted regions in the mouse and comprises the paternally expressed genes *Ins2*, *Igf2* and *Igf2as* in addition to the maternally expressed *H19* antisense RNA transcript. *Igf2* and *H19* share a common enhancer element, with imprinting mediated through the paternally methylated IC1 DMR (also called IC1 or *H19*-DMR) located between the *Igf2* and *H19* genes (Ferguson-Smith *et al.* 1993; Leighton *et al.* 1995b; Thorvaldsen *et al.* 1998; Tremblay *et al.* 1997). IC1 contains several recognition motifs for the zinc-finger DNA-binding protein CTCF, which acts as a functional insulator to block the effect of enhancer elements (Bell *et al.* 1999; Szabo *et al.* 2000) (summarised in Fig 1.10). Binding of CTCF to the hypomethylated maternal IC1 prevents interaction of the *Igf2* promoter with downstream enhancers, thus inactivating the maternal *Igf2* allele (Bell and Felsenfeld 2000; Kanduri *et al.* 2000; Szabo *et al.* 2000). Binding of CTCF also maintains the differential methylation of the domain and further acts to promote transcription of H19 (Engel *et al.* 2006; Schoenherr *et al.* 2003). In contrast, CTCF is unable to bind to the methylated paternal allele, thus in theory allowing the *Igf2* promoter to interact with enhancer elements and so enabling its transcription. The absence of CTCF permits methylation of the paternal chromosomal region, which renders the paternal H19 allele inactive. In addition, the different epigenetic modifications at IC1 result in altered chromatin arrangements of the two parental alleles, such that *Igf2* is located in an active chromatin structure on the paternal allele, but in a silent chromatin loop on the maternal copy (Murrell *et al.* 2004). Imprinting at IC1 is often described as a "boundary" or "insulator" model due to the action of the *H19*-DMR as a boundary element between *H19* and *Igf2*.

In addition to the differential methylation of IC1, a post-fertilisation paternally methylated DMR (DMR1) is located upstream of *Igf2* (Eden *et al.* 2001; Feil *et al.* 1994). The unmethylated maternal allele interacts with the repressor protein GCF2, which prevents transcription of *Igf2*. Methylation of the paternal DMR1 prevents binding of GCF2, thus allowing transcription of *Igf2* from the paternal allele (Eden *et al.* 2001). This mechanism acts independently of IC1, with maternal inheritance of DMR1 deletion resulting in biallelic expression of *Igf2*, whereas *H19* expression remains monoallelic (Constância *et al.* 2000).

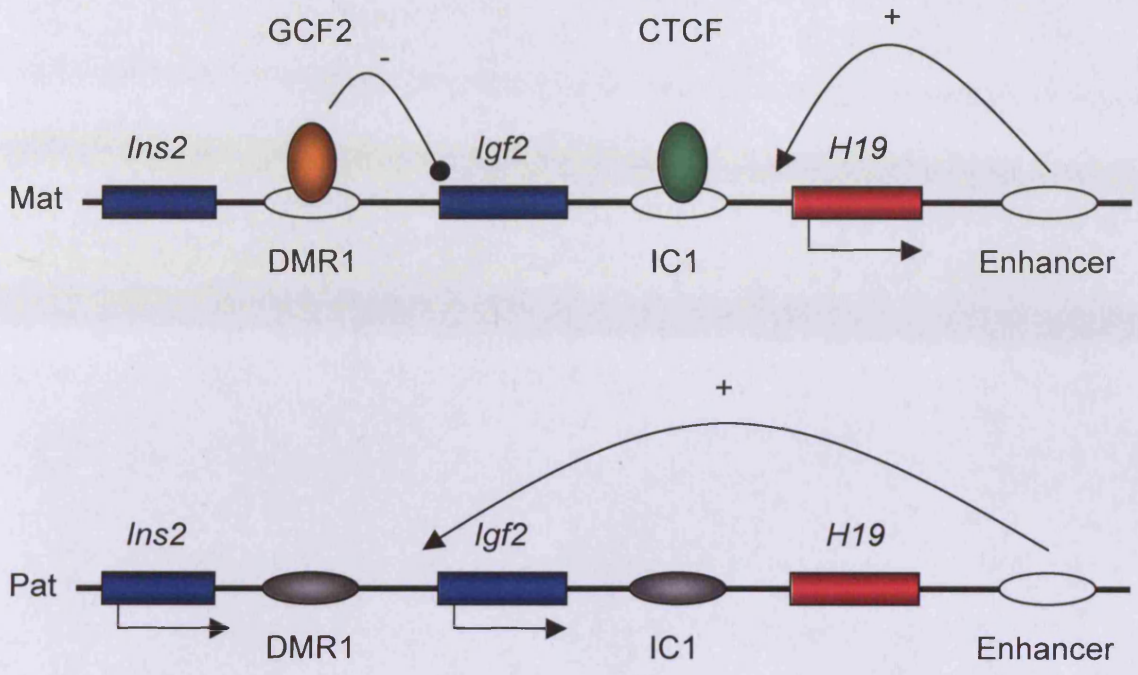


Figure 1.10: Control of imprinting at IC1

Imprinting at IC1 is regulated by the "enhancer blocking" or "insulator" mechanism. The DNA binding protein CTCF binds to the unmethylated maternal IC1 sequence, preventing interaction of *Igf2* with upstream enhancer elements and thus allowing maternal transcription of *H19*. CTCF cannot bind to the paternally methylated IC1 sequence, thus allowing *Igf2* to interact with enhancer elements. A second *Igf2*-specific level of control is provided by binding of the repressor protein GCF2 at the maternally unmethylated DMR1. Paternal methylation of this sequence prevents GCF2 binding, thus allowing *Igf2* transcription. Diagram not to scale

1.7.2 Control of imprinting at the mouse IC2 sub-domain

Transcription of *H19* is associated with repression of IC1 sub-domain genes on the same chromosome. *H19* expression is not however involved in the regulation of gene expression at the IC2 sub-domain. Maternal inheritance of a *H19* null allele results in biallelic expression of *Igf2* (Leighton *et al.* 1995a; Ripoche *et al.* 1997), whereas expression of *Ascl2*, *Cdkn1c* and *Kcnq1* remains monoallelic (Caspary *et al.* 1998). Monoallelic expression of genes within the telomeric IC2 sub-domain of mouse distal 7 is regulated by a mechanism independent of H19.

The mouse IC2 domain contains at least 11 genes that exhibit preferential expression of the maternal allele to varying degrees, including *Ascl2* (Guillemot *et al.* 1995), *Cdkn1c* (Hatada and Mukai 1995), *Phlda2* (Qian *et al.* 1997) and *Slc22a18* (Dao *et al.* 1998), all of which exhibit strong expression in the placenta. The domain also contains a paternally expressed ~60-kb ncRNA, *Kcnq1ot1*, which is transcribed from a promoter within intron 10 of *Kcnq1*. The *Kcnq1ot1* promoter is methylated on the maternal chromosome, forming a differentially methylated region referred to as *Kvdmr1*. Methylation of *Kvdmr1* prevents expression of *Kcnq1ot1*, whereas paternal expression of *Kcnq1ot1* is associated with silencing of the protein-coding genes in the domain (Smilinich *et al.* 1999) (summarised in Fig 1.11).

Two regions have been identified as essential for *Kcnq1ot1* expression: a ~1.9-kb region of *Kvdmr1* and a 244-bp region of the *Kcnq1ot1* promoter. Deletion of either of these regions causes loss of expression of *Kcnq1ot1* and biallelic expression of genes, including *Ascl2*, *Cdkn1c*, *Phlda2* and *Slc22a18* (Fitzpatrick *et al.* 2002; Mancini-Dinardo *et al.* 2006). Transcription initiation is insufficient for maintenance of monoallelic gene expression, with a truncated *Kcnq1ot1* transcript also associated with LOI of adjacent imprinted genes, indicating that a full-length *Kcnq1ot1* transcript is required for complete imprinting at the IC2 sub-domain (Mancini-Dinardo *et al.* 2006; Thakur *et al.* 2004). Additionally, *Cdkn1c* acquires *Kvdmr1*-dependent methylation of the paternal *Cdkn1c* promoter between E6.5 and E7.5. This post-fertilisation methylation is not required for initiation of the imprint, although it is probably required to maintain monoallelic expression of *Cdkn1c*, with *Dnmt* deficient mice exhibiting biallelic expression of *Cdkn1c* (Bhogal *et al.* 2004; Caspary *et al.* 1998).

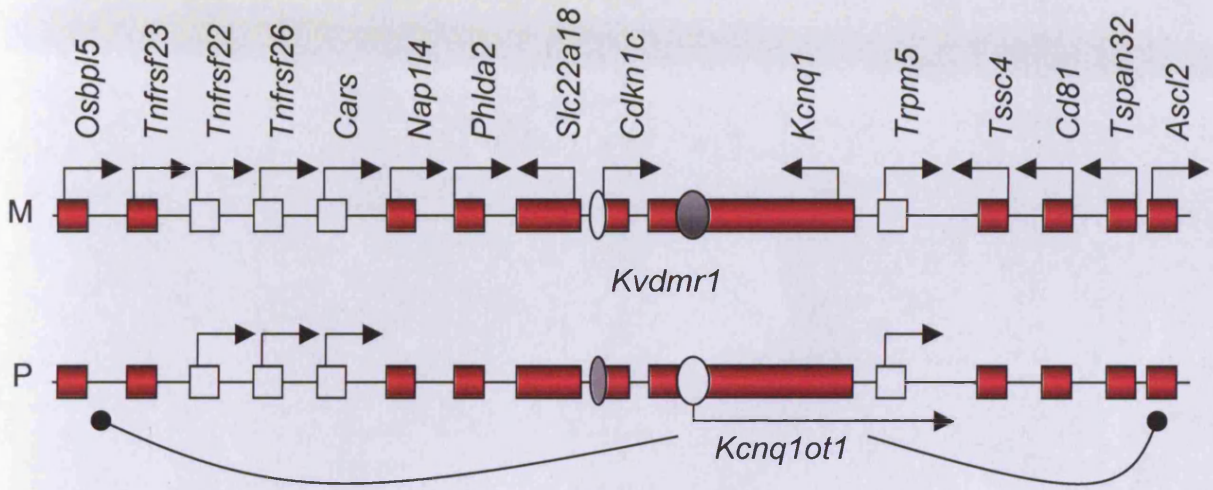


Figure 1.11: Imprinting at the mouse IC2 domain

Imprinting at the mouse IC2 domain is regulated by transcription of the ncRNA *Kcnq1ot1* from the paternal allele, which prevents transcription of adjacent genes from the paternal chromosome. Methylation of the maternal *Kcnq1ot1* promoter prevents transcription of the ncRNA and thus allows maternal expression of genes both up and downstream. Monoallelic expression of *Cdkn1c* is maintained by paternal methylation of the promoter in a *Kcnq1ot1* dependent manner. Diagram not to scale. M = Maternal allele; P = Paternal allele

Although classified as an imprinted gene, *Nap1l4/NAP1L4* only exhibits monoallelic expression in the mouse placenta (Engemann *et al.* 2000), with biallelic expression observed in the embryo (Paulsen *et al.* 1998) and all human tissues (Hu *et al.* 1996). Initially *Nap1l4* was presumed to mark the telomeric extent of the mouse IC2 sub-domain, with biallelic expression of the adjacent *Cars/CARS* in both mouse and human appearing to support this (Engemann *et al.* 2000). However, *Tnfrsf23*, which is located approximately 150-kb upstream of *Nap1l4* displays weak imprinting, with preferential expression of the maternal allele in some mouse tissues including the placenta (Clark *et al.* 2002). Additionally, expression analysis of *Kvdmr1* mutant mice revealed imprinted expression of *Osbpl5*, which is located adjacent to *Tnfrsf23* (Mancini-Dinardo *et al.* 2006). Thus, *Kvdmr1* controls the imprinting of genes that lie at least 200-kb outside of the previously defined IC2 region. Imprinted expression has not been reported for the three genes that lie between *Nap1l4* and *Tnfrsf23*.

1.7.3 Phenotypes associated with human 11p15.5 and mouse distal 7

The human overgrowth syndrome BWS is associated with aberrant expression of imprinted genes from 11p15.5 (Ping *et al.* 1989). BWS results from a variety of genetic and epigenetic alterations within this region, including paternal UPD of 11p15.5 (Henry *et al.* 1991), LOI of *IGF2* (Weksberg *et al.* 1993) and mutation of *CDKN1C* (Hatada *et al.* 1996). The most common alteration associated with BWS is loss of methylation at the maternal *KCNQ1OT1* promoter, which is predicted to result in biallelic expression of *KCNQ1OT1* and silencing of normally maternally expressed genes (Lee *et al.* 1999b). In contrast, maternal UPD of 11p15.5 (resulting in biallelic expression of genes including *CDKN1C*) and loss of methylation at IC1 (resulting in loss of *IGF2* expression) are associated with the growth restriction disorder Silver-Russell Syndrome (Fisher *et al.* 2002; Gicquel *et al.* 2005).

Uniparental disomy of two separate chromosomal regions is associated with embryonic lethality in the mouse due to the altered dosage of imprinted gene expression (reviewed in Cattanach and Beechey 1997). Paternal uniparental disomy of chromosome 12 (pUPD12) results in placentomegaly and late gestation embryonic death, whereas maternal uniparental disomy of chromosome 12 (mUPD12) is associated with perinatal lethality (Georgiades *et al.* 2000).

Similarly, mUPD7 causes placental deficiency, embryonic growth retardation and death by E16.5, whereas pUPD7 results in placental deficiency and restricted embryonic growth, with embryonic death by around E10.5 (McLaughlin *et al.* 1996; Searle and Beechey 1990). The altered dosage of several imprinted genes in these UPD models, with both loss of expression and over-expression, makes it impossible to determine the contribution of each gene to the phenotype. Investigations using single gene knockout mice have enabled the role of some genes to be investigated.

Embryonic lethality of pUPD7 conceptuses appears to be primarily associated with loss of *Ascl2*. *Ascl2* acts to prevent the differentiation of spongiotrophoblast cells into secondary giant cells, with *Ascl2* null placentae exhibiting an expanded giant cell layer and a complete loss of spongiotrophoblast (Guillemot *et al.* 1995; Guillemot *et al.* 1994; Scott *et al.* 2000). Similar to pUPD7 conceptuses, *Ascl2* null embryos die at ~E10.5 due to a similar placental defect (Guillemot *et al.* 1994; McLaughlin *et al.* 1996). The embryonic lethality associated with *Ascl2* null conceptuses was rescued by generating chimeras with tetraploid wild type embryos. The tetraploid wild type cells form extraembryonic tissue only, with viable embryos derived from diploid *Ascl2* null cells, confirming that the embryonic lethality is due to the lack of spongiotrophoblast and subsequent placental failure (Guillemot *et al.* 1994). The embryonic lethality of mUPD7 conceptuses has not been attributed to a single gene. Loss of the paternally expressed growth factor *Igf2* appears to contribute to the reduced embryonic and placental growth of mUPD7 embryos, with *Igf2*-null mice exhibiting smaller placentae and reduced birth weights (DeChiara *et al.* 1990; Ferguson-Smith *et al.* 1991). However, *Igf2* null mice are healthy and viable indicating a role for other genes in the lethality of mUPD7 (DeChiara *et al.* 1990). We recently reported that excess *Cdkn1c* in only a subset of tissues that endogenously express *Cdkn1c* is associated with embryonic lethality in BAC transgenic mice (Andrews *et al.* 2007). Thus, a combination of excess *Cdkn1c* and loss of *Igf2* expression may be causative of embryonic lethality in mUPD7 embryos. Interestingly, the opposite scenario of loss of *Cdkn1c* and LOI of *Igf2* generates a mouse model of BWS (Caspary *et al.* 1999).

Examination of the phenotype resulting from the loss of an imprinted gene is useful for assigning a function to a particular gene product. These models are

often found to represent useful tools for investigating human disease. In the case of imprinted genes, knockout models provide a useful indication of gene function, but provide less insight as to the advantage of acquiring an imprint at a specific gene or locus. Particular interest thus lies in generating models in which the effect of biallelic expression can be investigated. Not only will these models be useful for investigating human diseases such as BWS, they may enable a better understanding as to the function of imprinting.

Mice that inherit a paternal deletion of *Kvdmr* display biallelic expression of six maternally expressed genes, including *Cdkn1c*, *Phlda2*, *Slc22a18* and *Ascl2*. These mice have significantly lighter placentae, and are born up to 25% lighter than wild type littermates (Fitzpatrick *et al.* 2002; Salas *et al.* 2004). Maternal inheritance of the same deletion has no effect on gene expression or birth weight (Fitzpatrick *et al.* 2002). In contrast, maternal inheritance of a *H19* deletion results in excess *Igf2* and *Ins2*, and subsequent overgrowth of progeny that persists into adulthood (Leighton *et al.* 1995a). Similar to the UPD conceptuses however, such models result in the deregulation of several genes. One way to gain greater understanding of an individual imprinted gene is to generate single gene over-expression models. However, generation of such models is significantly more difficult than creating a knockout model, with no approach able to reactivate the silent allele of a single imprinted gene. In general, two approaches can be taken to generate mouse models for over-expression of imprinted genes. One is to directly disrupt the IC, resulting in LOI of all imprinted genes that are under the control of this element. Such mice have been generated for both the IC1 and IC2 sub-domains of mouse distal 7. An alternative approach is to introduce additional copies of imprinted gene(s), for example using BAC transgenes.

1.7.4 Cdkn1c

Cdkn1c (*p57^{Kip2}*) (*cyclin dependent kinase inhibitor 1C*) encodes a cyclin-dependent kinase inhibitor that is localised within the nucleus and inhibits G_1 cyclin-dependent kinases (CDKs), and prevents cell proliferation (Lee *et al.* 1995; Matsuoka *et al.* 1995). In the mouse, *Cdkn1c* is expressed in a range of tissues during embryogenesis, including skeletal muscle, neural tissue, lung, kidney, heart and the placenta (Nagahama *et al.* 2001; Westbury *et al.* 2001). Expression in adult tissues is restricted to the kidney and the cortex of the adrenal gland

(Nagahama *et al.* 2001). Tissue specific expression of the mouse *Cdkn1c* gene is controlled by a number of *cis*-elements, with embryonic enhancer elements located up to 225-kb downstream of *Cdkn1c*. Placental specific enhancers are yet to be identified, but are located at least either 50-kb upstream of *Cdkn1c* or over 225-kb downstream (John *et al.* 2001). Human *Cdkn1c* transcripts exist as either a 1.5-kb or a 7-kb mRNA transcript, with the 1.5-kb mRNA expressed at high levels in the placenta, at lower levels in skeletal muscle, heart, kidney and pancreas, and very low levels in the brain. The 7-kb transcript is observed only in skeletal muscle and the heart (Lee *et al.* 1995). In both humans and mice, *Cdkn1c* is generally expressed in cells as they enter terminal differentiation.

Cdkn1c null mice exhibit placentomegaly, with approximately twice the number of spongiotrophoblast and labyrinthine cells, although the number of both giant cells and glycogen cells remains unaffected (Takahashi *et al.* 2000a). Embryonic development in the absence of *Cdkn1c* expression was investigated in three separate mouse models, with variable reported phenotypes, potentially due to genetic background differences (Hagan *et al.* 2004). Zhang *et al.* (1997) deleted exons 1 and 2 of the *Cdkn1c* gene and reported phenotypes characteristic of BWS in humans, including cleft palate, with all mutant animals dying within 24 hours of birth. Yan *et al.* (1997) deleted exon 1 and around 50% of exon 2, resulting in various developmental defects including a shortened small intestine in all mutants and cleft palate in approximately 50% of mutants. Approximately 10% of these mutant animals survived to adulthood and did not display any developmental or growth abnormalities. Takahashi *et al.* (2000b) deleted the entire *Cdkn1c* coding region (exons 2 and 3), and also reported the survival of 10% of mutants. However, these animals displayed severe growth retardation and defective development of the male and female reproductive organs. We have recently reported that over-expression of *Cdkn1c* in a subset of embryonic tissues causes embryonic growth retardation that persists into adulthood. The placental phenotype could not be investigated in these animals as the enhancer for *Cdkn1c* placental expression is not present on the transgene (Andrews *et al.* 2007).

1.7.5 Igf2

One of the first imprinted genes to be described in mice was *Igf2* (*Insulin-like growth factor 2*), which encodes a potent mitogenic growth factor that regulates fetal and placental growth (DeChiara *et al.* 1990; DeChiara *et al.* 1991). *Igf2* is widely expressed in embryonic and placental tissues during gestation, with levels declining towards term (reviewed in Fowden 2003). In the mouse, *Igf2* is transcribed from four promoters; embryo-specific transcripts are expressed from promoters P1, P2 and P3, with placental *Igf2* transcribed from the P0 promoter (Feil *et al.* 1994; Moore *et al.* 1997).

Imprinting of *Igf2* was initially discovered through the observation that an *Igf2* null allele only caused a growth phenotype when inherited through the male germline (DeChiara *et al.* 1990; DeChiara *et al.* 1991). Maternal inheritance of the same disrupted allele had no growth consequences, whereas paternal inheritance resulted in a ~40% reduction in both embryonic and placental weights, from which adult mice did not recover. Cell number and content of glycogen cells was significantly reduced in *Igf2* null placentae, indicating a role of *Igf2* in glycogen cell differentiation and/or the synthesis of glycogen by this trophoblast sub-type (Lopez *et al.* 1996). Despite accounting for only approximately 10% of total placental *Igf2* transcripts (Moore *et al.* 1997), loss of placenta-specific *Igf2*P0 transcripts resulted in a comparable placental phenotype to *Igf2* null mice, with a 30% reduction in placental weight throughout mid to late gestation. In contrast, *Igf2* null embryos were growth restricted from early gestation and into adulthood, whereas *Igf2*P0 null embryos displayed progressive growth restriction from approximately E15.5, and caught up in weight compared to wild type animals by three months of age. The late gestation embryonic growth restriction of *Igf2*P0 null embryos seemingly occurs due to placental insufficiency (Constância *et al.* 2002). The delayed growth restriction may also be partially attributed to upregulation of nutrient transporter expression in *Igf2*P0 null placentae at E15.5. The mutant placenta was seemingly able to respond to the nutrient demands of the embryo by upregulating expression of *Slc2a3*, which encodes a glucose transporter, and *Slc38a4*, which encodes a member of the system A amino acid transporter family. The altered gene expression was associated with a compensatory increase in nutrient transport efficiency at E15.5,

although this compensatory effect was absent at E18.5. Embryonic growth restriction therefore occurred because the placenta was unable to continue to supply the necessary nutrients for normal growth. In contrast, such altered gene expression was not observed in *Igf2* null conceptuses, which possess similarly small placenta but with inherently smaller embryos also. It was proposed that the mutant placenta was able to supply the mutant embryo with sufficient nutrients to reach its genetic growth potential, without the need to increase nutrient transport (Constância *et al.* 2005; Constância *et al.* 2002; Sibley *et al.* 2004).

Over-expression of *Igf2*, either due to maternal inheritance of a disrupted *H19* allele or over-expression of the endogenous locus due to the presence of *Igf2* transgenes, resulted in placentomegaly and embryonic overgrowth of between 30% and 60%, which persists into adulthood (Eggenschwiler *et al.* 1997; Leighton *et al.* 1995a; Sun *et al.* 1997). Furthermore, deletion of the reciprocally imprinted *Igf2r* gene, which encodes an antagonist of Igf2 signalling, also results in placentomegaly and embryonic overgrowth, although *Igf2r*-null mice die soon after birth (Lau *et al.* 1994).

1.7.6 Phlda2

The maternally expressed gene *Phlda2* (*Pleckstrin homology-like domain family a member 2*) encodes a small, cytoplasmic protein of the specific function of which remains unknown (Frank *et al.* 2002; Qian *et al.* 1997). *Phlda2* is highly expressed in extraembryonic tissue, with the highest level of expression reported in the extraembryonic membranes and relatively lower expression in the placenta. Moderate levels of expression were reported in fetal liver and lower levels in fetal lung, kidney and limb buds. In the adult mouse, expression is only observed in the kidney. Expression of *Phlda2* in the placenta is detected in the ectoplacental cone following implantation, becoming restricted to the labyrinth layer by E10.5 (Frank *et al.* 1999). Between E12.5 and E14.5, the number of *Phlda2*-expressing cells declines significantly, restricted to the type II trophoblast cells and at lower levels in type III trophoblast cells. *Phlda2* is not expressed in the spongiotrophoblast or giant cells (Frank *et al.* 2002; Frank *et al.* 1999). Expression of human *PHLDA2* is more restricted, with high expression detectable only in extraembryonic tissue, and background expression observed in lung and

kidney of embryos and adults (Dunwoodie and Beddington 2002; Qian *et al.* 1997)

The mouse Phlda2 protein is 144 amino acids in length (152 in humans), comprising a single pleckstrin homology (PH)-like domain enclosed by short N- and C-termini (Frank *et al.* 1999; Qian *et al.* 1997). The PH domain is an amino acid sequence of between 100-120 amino acids that was first identified in the protein Pleckstrin (Haslam *et al.* 1993). Over 100 proteins have since been identified with homologous PH domains, most of which are implicated in signal transduction due to their affinity for various phosphatidyl inositol phosphate (PIP) molecules (reviewed inLemmon and Ferguson 2000). Similar to many PH-domain proteins, Phlda2 binds a wide range of PIP molecules with moderate affinity but low specificity, indicating a potential role in PIP signalling, but suggesting that this is either a broad role, or that the PIP molecule to which Phlda2 is specific is yet to be identified (Saxena *et al.* 2002). The presence of only a single functional domain within the protein suggests a role of Phlda2 as a signalling antagonist.

Phlda2 null mice exhibit placentomegaly with a disproportionate expansion of the spongiotrophoblast and an increased abundance of glycogen cells. However, no growth advantage was conferred to the embryo as a result of the larger placenta (Frank *et al.* 2002). Over-expression of *Phlda2* and *Slc22a18* results in smaller placenta, and a subsequent reduction in embryonic growth. Restoration of *Phlda2* gene dosage to normal in *Kvdmr1* null mice revealed that the reduced placental weight in this model was due in part to excess *Phlda2* (Salas *et al.* 2004).

1.7.7 Slc22a18

The maternally expressed *Slc22a18* (solute carrier family 22 (organic cation transporter), member 18) (formerly *Impt1*) encodes an organic cation transporter molecule that is highly expressed in the mouse placenta (Dao *et al.* 1998). The mouse *Slc22a18* gene encompasses 11 exons and encodes a protein of 410 amino acids in length, with a similar organisation existing in the human *SLC22A18* gene, which encodes a protein of 424 amino acids. Expression of *Slc22a18* is restricted to tissues with transport function, with high expression levels in the placenta yolk sac, fetal kidney, liver and intestine (Dao *et al.* 1998). Although only a general role of Slc22a18 has been described in organic cation

transport, reduced levels of *Slc22a18* expression are associated with impaired kidney function in *Aprt*-deficient mice (Tzortzaki *et al.* 2003).

1.8 Intrauterine growth restriction and fetal programming

Birth weight is routinely used to assess the general health of a newborn baby at delivery. Full-term infants with a birth weight below the 10th centile are generally referred to as small for gestational age (SGA), and by definition account for 10% of all births (Lubchenco *et al.* 1963). SGA infants can however be further classified according to the cause of their small size. Some SGA infants are inherently small, having achieved their predetermined, genetic growth potential (reviewed in Bamberg and Kalache 2004). However, a significant proportion, estimated at between 3% to 7% of all infants fail to achieve their genetic growth potential as a consequence of a variety of genetic or environmental factors. Such infants are said to have undergone intrauterine growth restriction (IUGR) (reviewed in Brodsky and Christou 2004). Furthermore, there are some infants whose birth weight falls in the normal range, but are nonetheless growth restricted, having failed to achieve their genetic growth potential (Blair 1994; Monk and Moore 2004). The terms IUGR and SGA are sometimes used synonymously in the literature, however they are not interchangeable, with IUGR referring to a failure to achieve the genetic growth potential and SGA describing an infant with birth weight below the 10th centile for its gestational age (Monk and Moore 2004). Thus, not all SGA infants have undergone IUGR, likewise, not all IUGR infants are SGA (Sifianou 2006).

IUGR may result from a variety of fetal, placental or maternal factors. Despite a significant proportion of IUGR cases remaining idiopathic, fetal factors, such as chromosomal abnormalities or multiple gestations (i.e. twins or triplets) are predicted to account for approximately 20% of cases. Maternal factors, including general health, drug or alcohol consumption, and in particular placental factors generally referred to as "utero-placental insufficiency" account for the remaining 80% of cases (reviewed in Brodsky and Christou 2004; and Lin and Santolaya-Forgas 1998). In the Western world, placental insufficiency is the most common cause of IUGR (Henriksen and Clausen 2002), resulting from insufficient trophoblast invasion, poor maternal or fetal blood flow, or other defects with placental structure or function (reviewed in Mandruzzato *et al.* 2008).

IUGR can be classified as either type I (symmetric), or type II (asymmetric), assessed by the ratio of head circumference to abdominal circumference (HC:AC) (Campbell and Thoms 1977). Around 20-30% of IUGR infants are symmetrically growth restricted, characterised by a proportional growth restriction of the head and body, with a HC:AC ratio in the normal range. Symmetric IUGR is frequently associated with fetal chromosomal abnormalities with the onset of fetal growth restriction during the first or second trimester. The remaining 70-80% of IUGR cases are asymmetric, exhibiting an increased HC:AC ratio due to a relative sparing of brain growth in comparison to the body. Such asymmetric growth restriction tends to result from placental insufficiency affecting embryonic growth during the third trimester (Lin *et al.* 1991; Lumbers *et al.* 2001; Monk and Moore 2004; and reviewed in Platz and Newman 2008). Post-natal catch up growth is observed in 70-90% of IUGR infants, and is generally complete by two years of age (Hokken-Koelega *et al.* 1995; Leger *et al.* 1997; Ong *et al.* 2000). Such catch up growth tends to be associated with asymmetrically growth restricted infants, resulting from a return to normal growth kinetics following removal of the growth restrictive factor acting *in utero* (Adair 1989; Fay and Ellwood 1993; Villar *et al.* 1984).

IUGR is associated with a 10 to 20-fold increased risk of perinatal lethality (Buck *et al.* 1989; Dobson *et al.* 1981; Kok *et al.* 1998; McIntire *et al.* 1999; Richardus *et al.* 2003). Epidemiological studies in human populations have reported increased occurrence of the "metabolic syndrome" in individuals that were born with a low birth weight. Metabolic syndrome is a multifactorial condition characterised by the presence of at least three key factors, including central (or visceral) obesity, insulin resistance, hypertension, dyslipidemia, elevated plasma triglyceride levels and elevated HDL levels, all of which are risk factors for cardiovascular disease (CVD) and Type 2 Diabetes (reviewed in Alberti *et al.* 2005). Low birth weight has been associated with increased rates of CVD (Barker *et al.* 1993; Barker and Osmond 1986; Osmond *et al.* 1993), hypertension (Barker *et al.* 1990) and impaired glucose tolerance (Hales *et al.* 1991). There is conflicting data as to whether low birth weight is also associated with increased obesity in human studies. Some studies report no association (McNeely *et al.* 2007), whereas others suggest a positive correlation between low birth weight and central adiposity in both adults (Law *et al.* 1992; Loos *et al.* 2001) and

children (Barker *et al.* 1997; Ong *et al.* 2000). Additional studies have reported reduced lean mass, although with no effect on fat mass in individuals that were born small, resulting in a relative increase of body fat proportion (Gale *et al.* 2001; Hediger *et al.* 1998). Further studies and a re-evaluation of existing data identified post-natal catch up growth as an additional risk factor for metabolic syndrome. Data from the "Helsinki cohort", consisting of over 7,000 men and women born in Helsinki between 1934-44, indicated that the risk of developing metabolic syndrome was increased in low birth weight infants that displayed post-natal catch up growth (Eriksson *et al.* 2000; Eriksson *et al.* 1999; Forsen *et al.* 2000; Forsen *et al.* 1999). In an independent study, low birth weight infants that displayed catch up growth by two years of age were more likely to be obese at five years of age (Ong *et al.* 2000).

1.8.1 Rodent models of IUGR

A number of animal models of IUGR exist, which demonstrate components of metabolic syndrome to varying degrees. Uterine artery ligation of the rat during late gestation results in IUGR, with a 15% reduction in birth weight. IUGR animals displayed catch up growth and became obese and developed glucose intolerance by 6 months of age (Simmons *et al.* 2001). Dietary induced models of IUGR include the maternal low protein model, in which pregnant dams are fed an isocaloric diet containing less than 50% of the protein in standard diet during pregnancy and lactation. Pups born to protein-restricted mothers have a significantly reduced birth weight, with catch up growth displayed by those pups suckled by standard chow fed dams (Desai *et al.* 1996). Male offspring born to protein restricted dams developed insulin resistance by 17 months of age (Petry *et al.* 2001). Similarly, female offspring born to protein restricted dams displayed hyperinsulinaemia at 21 months, possibly indicative of the development of insulin resistance (Fernandez-Twinn *et al.* 2005).

Similar to the protein restriction model, a calorie-restricted diet during gestation induces a low birth weight, although animals did not exhibit catch up growth when suckled by *ad libitum* fed dams. Subsequent weaning onto a hypercaloric resulted in development of hypertension and hyperinsulinaemia. Although the body weight of calorie-restricted animals remained lower than control animals, the retroperitoneal fat pad mass relative to body weight was

significantly increased in both standard chow fed and hypercaloric diet fed IUGR rats (Vickers *et al.* 2000).

The importance of catch up growth for the programming of adult obesity was subsequently demonstrated in both the calorie and protein restricted rat models. Whereas protein or calorie restriction during gestation and lactation yielded growth-restricted offspring that did not display catch up growth or obesity, over-feeding during lactation induced obesity in both models. Exposure to a hypercaloric diet from weaning further exacerbated the weight gain, with calorie-restricted animals gaining significantly more weight than protein restricted animals (Bieswal *et al.* 2006).

A similar model of IUGR has been applied to mice, with pregnant dams fed an 8% protein diet during gestation and offspring cross-fostered to dams fed either a standard 20% protein diet (recuperated) or the 8% protein diet (low protein diet). Recuperated mice were born significantly lighter but exhibit rapid catch-up growth whereas the low protein fed offspring were born small and remain small throughout their lives (Ozanne *et al.* 2004). Feeding recuperated animals a highly palatable "cafeteria" diet from weaning exacerbated weight gain and reduced average longevity. Longevity was improved by maintaining IUGR pups on a low protein diet during lactation (Ozanne and Hales 2004; Ozanne *et al.* 2004).

1.8.2 The "Thrifty Phenotype" hypothesis

The relationship between birth weight and adult health and disease led David Barker and colleagues to propose the "thrifty phenotype hypothesis", suggesting that poor fetal and neonatal nutrition leads to permanent alterations to organs including the pancreas (Hales and Barker 1992). This so-called "fetal programming" is proposed to occur in response to nutrient deprivation during critical developmental windows, primarily late gestation and during suckling (reviewed in McMillen and Robinson 2005). Exposure to a nutrient restricted environment *in utero* results in an adaptive metabolic response in organs such as the pancreas and an increased blood flow to the brain at the expense of other organs (Hales and Barker 1992; Lumbers *et al.* 2001). Such adaptations are irreversible but essential for the immediate survival of the fetus. The hypothesis proposes that these alterations would continue to be advantageous if nutrient

restriction persisted in the post-natal environment, as predicted by exposure *in utero*. However, in instances when the post-natal environment is nutrient rich in comparison to the *in utero* environment, the "programmed" metabolism is unable to respond adequately, eventually resulting in diabetes and obesity (Hales and Barker 1992, 2001).

Despite an established link between IUGR and metabolic syndrome, the mechanisms of fetal programming that underlie this association are only now beginning to be understood. Epigenetic alterations are proposed to play a key role in the adaptive mechanism through regulation of gene expression in response to metabolic needs (Burdge *et al.* 2007; Gluckman *et al.* 2007; Godfrey *et al.* 2007). Imprinted genes, which are epigenetically regulated and control embryonic and placental growth, are potential candidates in both IUGR and fetal programming. Indeed, several imprinted genes have been implicated in IUGR in mice, including *Igf2* (Constância *et al.* 2002; DeChiara *et al.* 1990) and *Cdkn1c* (Andrews *et al.* 2007). Loss of *Igf2* and over-expression of *Cdkn1c* result in symmetric growth restriction from which adult animals do not recover, whereas loss of *Igf2* in the placenta only, causes embryonic growth restriction with post-natal catch up growth. *Phlda2* is another imprinted gene in the same imprinted domain and has been implicated in IUGR in mouse models, with over-expression causing reduced placental growth resulting in embryonic growth restriction at E16.5 (Salas *et al.* 2004). *PHLDA2* has recently been reported to be upregulated in human placentae of IUGR and lower birth weight infants, although no alteration was reported for *IGF2* (Antonazzo *et al.* 2008; Apostolidou *et al.* 2007; McMinn *et al.* 2006). Over-expression of *Phlda2* in the mouse placenta may thus represent a model for a known human scenario, which can be used to further investigate IUGR and fetal programming.

1.9 Aims and objectives

Two approaches have been undertaken to investigate the function of imprinted genes. The first is to generate knockout mouse models by specifically targeting the actively expressed allele. The mutant allele is subsequently transmitted through the appropriate germline such that progeny inherit a disrupted allele on the normally active chromosome. The disrupted allele would be silenced by transmission through the alternative germline and would thus not be associated

with any phenotype. Such knockout models are useful in identifying gene function, but provide less insight as to the advantage of monoallelic expression over biallelic expression. To investigate the potential rationale for actively reducing the amount of gene product produced, an alternative model in which expression levels are restored to biallelic levels or above can be used. Such models can be generated for some genes of an imprinted domain by targeting the ICR, thus resulting in altered expression of all genes under its control. However, this would result in two-fold expression of some genes with loss of expression of others. Thus, there is no direct mechanism to generate biallelic expression of a single gene in a complex imprinted domain. One approach that has been utilised is the generation of mice that possess additional copies of the imprinted gene on transgenes. Such an approach relies upon the availability of appropriate genomic constructs that contain an intact copy of the gene of interest in addition to any elements essential for transcription. Furthermore, the resulting transgenic mice may possess multiple copies of the transgene, or exhibit de-regulated expression of the transgene, and thus may still not represent true models of LOI.

The transgenic mouse model that was primarily utilised during this investigation (line 10-15) possesses three copies of a modified BAC transgene that spans the genes *Phlda2*, *Slc22a18* and *Cdkn1c*, but with *Cdkn1c* inactivated by insertion of a *β-galactosidase* reporter (John *et al.* 2001), thus isolating the role of *Phlda2* and *Slc22a18* in development. Preliminary investigations into this transgenic model identified a significant reduction in placental weight at E14.5 and E16.5, with a subsequent reduction in embryonic weight at E16.5. This reduced placental weight was indirectly attributed to a reduction in the spongiotrophoblast component (Salas *et al.* 2004).

Further to the initial observation that over expression of *Phlda2* results in a placental defect, the primary aims of this investigation were to perform a detailed characterisation of the placental phenotype associated with over expression of *Phlda2* and to determine any associated post-natal consequences. The investigation focused primarily on characterising the phenotype on a pure 129/Sv genetic background using BAC transgenic mice that over express the imprinted genes *Phlda2* and *Slc22a18*. The effect of genetic background on placental phenotype was examined on a 129/Sv x C57BL/6 background. Additionally, as part of this study a second independent transgenic line was generated and

utilised to confirm the observed placental phenotypes on a 129/Sv x C57BL/6 genetic background. The effect of transgene copy number was examined on two additional transgenic lines that possess either one or two copies of the transgene.

The placental phenotype was characterised by utilising a number of approaches and techniques. Gene expression levels in transgenic placentae were compared to those of wild type by microarray and Quantitative Reverse Transcription PCR (qPCR). The gross placental morphology was examined by Haematoxylin and Eosin (H&E) staining of midline placental sections, from which spongiotrophoblast and labyrinth areas were measured. Furthermore, the relative abundance of glycogen cells was assessed by using periodic acid-Schiff (PAS) staining. The spatial expression pattern of various placental markers was examined using *in situ* hybridisation. Probes were utilised for genes including *Phlda2* and *Slc22a18*, which are expressed from the transgene, *Tpbpa*, which specifically marks spongiotrophoblast and the giant cell markers *Pl1* and *Pl2*. The primary placental phenotypes identified in transgenic placentae from line 10-15 on a pure 129/Sv background were also examined on the 129/Sv x C57BL/6 genetic background and in additional independent transgenic lines.

Post-natal characterisation was performed entirely on mice from line 10-15 on a pure 129/Sv genetic background. Organ weights were analysed from neonatal animals, and a cohort of animals was weighed over the course of a year. Subsets of male and female mice from this cohort were subjected to glucose tolerance tests at 10 weeks, 6 months and 1 year of age. Furthermore, additional animals were dissected at these same ages to examine adiposity at two defined depots. Gross organ morphology was examined by H&E staining of organs from neonatal and adult animals, with specific measurements of some organs performed using computer software. A separate cohort of animals was also subjected to MRI scanning to investigate the potential for the use of this technique in future longitudinal studies of fat deposition.

Chapter 2:

Materials and Methods

2.1 Transgenic mouse lines

Several transgenic mouse lines were utilised during the course of this investigation. The majority of placental and post-natal characterisation studies were performed using mice from line 10-15(*Phlda2*) (henceforth line 10-15) maintained on a pure 129/Sv genetic background. These mice possess three copies of a BAC transgene that encompasses a discrete region of the mouse distal 7 imprinted region (Fig 2.1A). The transgene contains intact copies of the imprinted genes *Phlda2* and *Slc22a18*. A third gene, *Cdkn1c*, is inactivated by the insertion of a *β-galactosidase* reporter construct (John *et al.* 2001) (Fig 2.1B). Transgenic mice from line 10-15 were also maintained on a C57BL/6 background, and utilised for the characterisation of the placental phenotype on a 129/Sv x C57BL/6 genetic background. In all such instances, the genetic background of the female is first, followed by the genetic background of the male. An independent line named 10-10(*Phlda2*) (henceforth line 10-10), containing an undetermined number of the same modified BAC transgene, was generated as part of this study and used to examine the placental phenotype in an independent transgenic line to 10-15. Transgenic mice were maintained on the C57BL/6 background, with placental characterisation performed on a 129/Sv x C57BL/6 genetic background.

The placental phenotype was also examined in a single copy transgenic line (5D3) and on a two-copy transgenic line (5A4). Both lines 5D3 and 5A4 possess an unmodified version of the BAC transgene, with an intact copy of the *Cdkn1c* gene (Fig 2.1C). Placenta-specific enhancer elements for *Cdkn1c* are not present on the transgene, thus *Cdkn1c* is not expressed from the transgene in the placenta (John *et al.* 2001). *Cdkn1c* is however expressed from the transgene in a subset of embryonic tissues that normally express *Cdkn1c*, including the lung epithelium and kidney, but not muscle or cartilage (John *et al.* 2001). Lines 10-15, 10-10, 5D3 and 5A4 thus differ only in the gene dosage of *Phlda2* and *Slc22a18* in the placenta, with *Cdkn1c* not expressed in the placenta of any line. Due to the previously reported embryonic lethality associated with maintaining lines 5D3 and 5A4 on the 129/Sv background (Andrews *et al.* 2007), both lines were maintained on the C57BL/6 background and placental phenotype examined on a 129/Sv x C57BL/6 genetic background.

To analyse the individual contribution of *Slc22a18* over-expression to the placental phenotype, transgenic males from the single copy transgenic line 5D3 were mated with females of the *Phlda2*^{Loxp} (henceforth *Phlda2* null or *Phlda2*$^{-/+}$) line to generate "double transgenics". Such conceptuses possess a single copy of the unmodified transgene and a maternally inherited *Phlda2* null allele, such that *Phlda2* gene dosage was returned to normal, whereas *Slc22a18* remains over-expressed. Generation of the *Phlda2* null line was described by Frank *et al.* (2002), with homozygous stud males maintained on the C57BL/6 background.

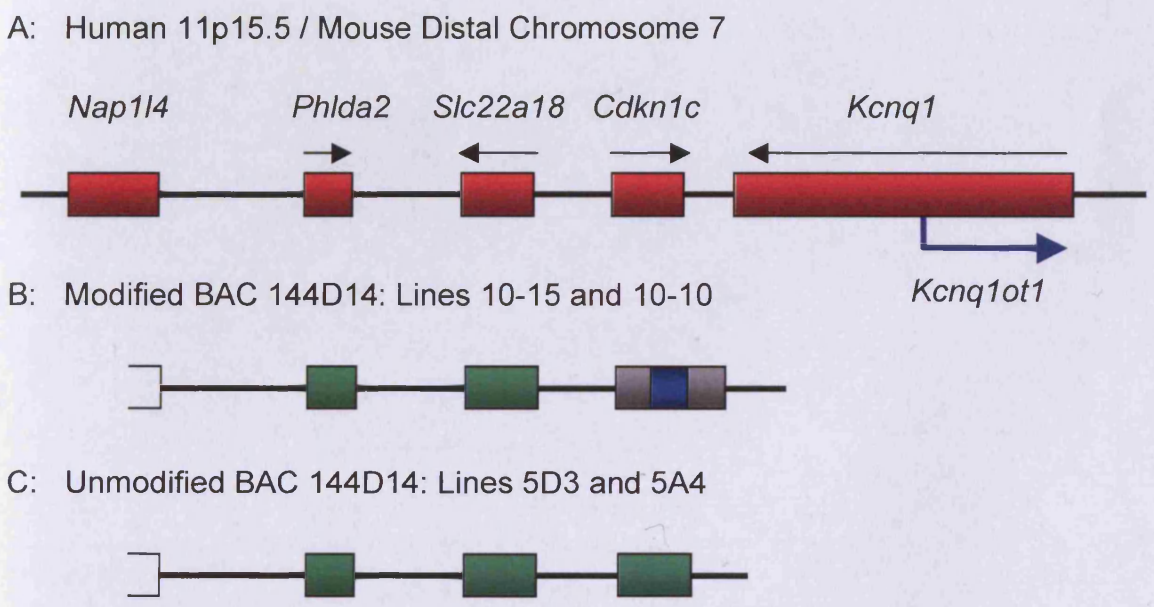


Figure 2.1: Structure of modified and unmodified BAC transgenes

A: Organisation of imprinted genes at mouse distal 7. B: Unmodified 85-kb BAC transgene containing the intact *Phlda2*, *Slc22a18* and *Cdkn1c* genes. C: Modified version of the 85-kb BAC transgene, including reporter gene *LacZ* (blue). *Cdkn1c* expression is disrupted by insertion of a *β-galactosidase* reporter gene. Maternally expressed genes are in red, the paternally expressed non-coding RNA transcript *Kcnq1ot* is shown by a blue arrow. Non-imprinted genes are green. The transgene does not undergo imprinting and thus transgenic copies of *Phlda2*, *Slc22a18* and *Cdkn1c* are shown in green.

2.1.1 Generation 10-15, 5D3 and 5A4 transgenic mouse lines

Generation of lines 10-15, 5D3 and 5A4 was described in John *et al.* (2001). Briefly, a 129/Sv gridded pBeloBAC library (Genome Systems, St Louis) was hybridised with a probe for *Cdkn1c*, resulting in the identification of the clone 144D14. This fragment extends 65-kb upstream of *Cdkn1c* and 20-kb downstream, encompassing the genes *Slc22a18*, *Phlda2* and part of *Nap1l4* in addition to *Cdkn1c* (Fig 2.1C). BAC DNA was linearised and ES stem cell lines were generated by co-electroporation with *PGK-neo*. Transgene copy number was determined by Southern blotting of genomic DNA from independent lines, with comparison of band intensity to the endogenous locus by phosphorimaging. Lines containing 1-6 copies of the BAC transgene were used to ultimately generate several lines, including 5D3 (single copy) and 5A4 (two copy).

The BAC clone was modified by homologous recombination of a *β-galactosidase* reporter construct (*p57Kip2-IRESβgeoloxPalkP*) into the *Cdkn1c* locus to report transgenic *Cdkn1c* expression. This modification also inactivated *Cdkn1c* on the BAC. Line 10-15 was generated from this construct by pronuclear injection into F_1 C57BL/6 x CBA embryos. Lines 10-15, 5D3 and 5A4 were generated at Cambridge University by Dr John.

2.1.1 Generation 10-10 transgenic mouse line

Transgenic line 10-10 was generated at Cardiff University using the same modified BAC transgene present in line 10-15. BAC DNA was prepared by Dr John and transgenic founder animals generated by pronuclear injection of linearised BAC DNA into C57BL/6 x CBA F_1 hybrid eggs. Pronuclear injection was performed at the Cardiff Transgenic Production Facility.

A single transgenic male was identified from 76 live born pups following pronuclear injection. This male was mated with a wild type C57BL/6 female by the Transgenic Production Facility to generate G2 C57BL/6 progeny, of which one male (of three) and two females (of four) were transgenic. The transgenic animals were returned to the conventional transgenic facility, where the male was mated with a wild type C57BL/6 female to generate G3 C57BL/6 progeny. The 7 progeny from this mating consisted of five females, which were culled prior to genotyping and two males, of which one was transgenic. Line 10-10 was further

backcrossed onto the C57BL/6 background as described in Section 2.2.1 until transgenic males from at least G6 were obtained.

2.2 Maintenance and mating of transgenic mice

All animals were maintained on a 12-hour light-dark cycle, under non-barrier conditions according to Home Office regulations, and fed *ad libitum* with standard rodent feed (Harlan, Oxfordshire) and tap water. Five transgenic mouse lines maintained on two genetic backgrounds were utilised during the course of this investigation. All over-expression transgenic lines were maintained by transmission of the transgene(s) from hemizygous males in a mating with wild type females. Transgenic males for line 10-15 were maintained separately on both the 129/Sv and C57BL/6 genetic backgrounds. Transgenic males for lines 10-10, 5A4 and 5D3 were maintained only on a C57BL/6 background. The *Phlda2* null line was maintained on a pure C57BL/6 background. Stocks of wild type C57BL/6 and 129/Sv females were maintained in the conventional transgenic facility at Cardiff University. An outline of the maintenance of transgenic lines is presented in Section 2.2.2.

2.2.1 Backcross to C57BL/6 background

Line 10-15 was maintained on the 129/Sv background for greater than 15 generations, and was thus considered pure 129/Sv. Lines 5D3 and 5A4 were previously maintained on the 129/Sv x MF1 background. Embryonic lethality was observed on both lines as the contribution of the 129/Sv background increased (Andrews *et al.* 2007). For this work, lines 5D3 and 5A4 were backcrossed to the C57BL/6 background until no lethality was observed. Line 10-10 was generated by pronuclear injection of BAC DNA into C57BL/6 x CBA F_1 hybrid eggs, and backcrossed to the C57BL/6 background.

To backcross each transgenic line, confirmed transgenic males were mated with a wild type C57BL/6 female, to yield live born pups. Male progeny were earmarked and genotyped as described in Section 2.3.1, with wild type males and all females killed prior to weaning. At four weeks of age, transgenic males were weaned to separate cages, and at six weeks of age were mated with a wild type C57BL/6 female. This procedure was repeated for at least six cycles, to establish transgenic males of at least generation 6 C57BL/6 (G6).

Backcrossing to the C57BL/6 background was continued beyond G6, although experimental conceptuses were generated using animals from G6 and beyond. Table 2.1 shows the relative genetic contribution of each genetic background at each generation.

Mating		Progeny	Genetic contribution	
Male	Female	generation	129/Sv	C57BL/6
129/Sv	C57BL/6	G1	50%	50%
G1 C57BL/6	C57BL/6	G2	25%	75%
G2 C57BL/6	C57BL/6	G3	12.5%	87.5%
G3 C57BL/6	C57BL/6	G4	6.2%	93.8%
G4 C57BL/6	C57BL/6	G5	3.1%	96.9%
G5 C57BL/6	C57BL/6	G6	1.6%	98.4%
G6 C57BL/6	C57BL/6	G7	0.8%	99.2%
G7 C57BL/6	C57BL/6	G8	0.4%	99.6%

Table 2.1: Relative contribution of genetic background after successive generations of backcrossing

The table shows the relative contribution of 129/Sv and C57BL/6 genetic backgrounds over the course of eight generations of backcrossing from a pure 129/Sv background to the C57BL/6 background.

2.2.2 Maintenance of stock animals

Stock animals for all transgenic lines were kept to an absolute minimum, with up to six transgenic males maintained for each line, dependent upon the level of usage. Stud males were used for breeding up to approximately one year of age, or until breeding efficiency became noticeably reduced. Stud males were renewed by mating a transgenic male with a wild type 129/Sv or C57BL/6 female as required, genotyping male progeny (see Section 2.3.1) and selecting transgenic animals for subsequent breeding. Wild type females were mated from 6 weeks of age until 6 months or until average litter size fell below five. All transgenic lines yielded transgenic over-expression animals in approximate Mendelian ratios, with 50% wild type and 50% transgenic animals born.

The primary use of the *Phlda2* null line was to cross with line 5D3 to observe the effect of returning *Phlda2* gene dosage to normal. Due to the

imprinted nature of *Phlda2*, female transmission of the *Phlda2* null allele was essential in experimental conceptuses. To minimise animal numbers, heterozygous *Phlda2* null females were generated from matings between homozygous *Phlda2* null males with wild type C57BL/6 females. Two homozygous C57BL/6 *Phlda2* null males were kindly provided by Miguel Constância of the University of Cambridge. All progeny of the first litter born to each stud were genotyped to confirm 100% transmission of the null allele; subsequently, no genotyping was performed on progeny from confirmed stud animals. All male progeny were culled, except when required to mate with a *Phlda2* null female to generate replacement homozygous stud males. Female progeny were weaned at four weeks of age, and mated with 5D3 stud males between 6 and 10 weeks of age as required.

2.2.3 Generation of conceptuses and experimental mice

Placental and embryonic phenotype characterisation was performed using mice from five transgenic lines on both pure 129/Sv and 129/Sv x C57BL/6 genetic backgrounds. Conceptuses on a pure 129/Sv background were generated by mating a transgenic male on the 129/Sv background with a wild type female also on the 129/Sv background. Conceptuses on a 129/Sv x C57BL/6 genetic background were generated by mating a transgenic male from at least G6 C57BL/6 with a wild type female on the 129/Sv background thus generating F1 129/Sv x C57BL/6 progeny. Transgenic male mice were mated with a wild type (or *Phlda2* null) female and females checked each morning for the presence of a vaginal plug. The day of plug discovery was recorded as embryonic day 0.5 (E0.5). Females were killed and embryos dissected at the appropriate stage as described in Section 2.4.

Post-natal characterisation was performed using mice from line 10-15 on a pure 129/Sv background. These mice were generated from mating hemizygous stud males with wild type 129/Sv females, allowing the birth of progeny, performing genotyping as described in Section 2.3.1 and weaning offspring at four weeks of age. A maximum of five animals were co-housed, with no experimental animals housed alone. All cages contained a mixture of both wild type and transgenic animals, restricted where possible to animals from the same

litter. Male animals from different litters were not mixed, and litters that contained only one male were not used for experimental purposes.

To investigate the relative contribution of *Slc22a18* and *Phlda2* to the observed placental phenotype, *Phlda2* gene dosage was restored to normal by crossing the *Phlda2* null line with the single copy over-expression line 5D3. A hemizygous 5D3 male was mated with a heterozygous *Phlda2*$^{-/+}$ female, which was expected to yield four potential genotypes in equal Mendelian ratios, as depicted by the Punnett square in Table 2.2. Both 5D3 males and *Phlda2* null females were maintained on the C57BL/6 background, and thus all conceptuses were at least G7 C57BL/6.

		Maternal alleles	
		Wild type	***Phlda2* null**
Paternal alleles	**Wild type**	Wild type	*Phlda2* null
	Transgene	Transgenic	*Phlda2* null & Tg

Table 2.2: Punnett square for mating 5D3 male with *Phlda2* null female

The Punnett square shows the four expected genotypes and ratios of progeny generated from a cross between transgenic males of line 5D3 with *Phlda2* null females. Each genotype would be expected to occur with a 25% frequency.

A summary of all breeding scenarios utilised during the course of this investigation is presented in Table 2.3. The first two rows show the use of wild type male and female mice from lines 129/Sv and C57BL/6 to produce wild type females for mating with transgenic males. Wild type males were occasionally retained from these litters to replenish stud males in maintaining these colonies. The table is subsequently organised according to the transgenic line, followed by the genetic background of transgenic males used for breeding from each line. These transgenic males were mated with wild type females of the designated genetic background to generate experimental animals either at various gestational stages ("embryos and placentae" in table), or for adult animals ("experimental adults"). Mating scenarios used for backcrossing specific transgenic lines are designated by the purpose of "stud males".

Line	Genetic background of:		Usage
	Transgenic male	Wild type female	
129/Sv	129/Sv	129/Sv	Wild type females
C57BL/6	C57BL/6	C57BL/6	Wild type females
10-15	129/Sv	129/Sv	Embryos and placentae Neonate weights Experimental adults Stud males
	C57BL/6	129/Sv	Embryos and placentae
		C57BL/6	Stud males
10-10	129/Sv	129/Sv	Stud males
	C57BL/6	129/Sv	Embryos and placentae
		C57BL/6	Stud males
5A4	C57BL/6	129/Sv	Embryos and placentae
		C57BL/6	Stud males
5D3	C57BL/6	129/Sv	Embryos and placentae
			Neonate weights
		C57BL/6	Embryos and placentae Stud males
		C57BL/6 (*Phlda2* null)	Embryos and placentae
Phlda2 null	C57BL/6	C57BL/6 (*Phlda2* null)	Homozygous stud males

Table 2.3: Summary of mouse breeding and usage

The table summarises all matings that were set up for each transgenic line according to genetic background and intended usage. The genetic background of the stud male is listed under "Stud" and the genetic background of the female is listed under "female". All females were wild type, unless stated otherwise. The broad intended purpose of the breeding is listed under the column "Usage".

2.3 Genotyping of transgenic mice

Two methods were utilised in order to determine the genotype of stock or experimental animals. All transgenic lines could be genotyped by Polymerase Chain Reaction (PCR) amplification of specific sequences from either tail biopsies or yolk sac DNA. Additionally, the presence of the *β-galactosidase* reporter construct in lines 10-15 and 10-10 allowed conceptuses from these transgenic lines to be genotyped by *β-galactosidase* staining of the embryo. All experimental animals were genotyped prior to weaning and genotype confirmed from a second tail biopsy post-mortem.

2.3.1 Genotyping by Polymerase Chain Reaction

PCR is routinely used to genotype genetically modified mice. Primers are designed to amplify a specific sequence of DNA, the size of which (or alternatively the presence or absence of a product) is used to determine genotype. Two PCR protocols were utilised to genotype animals used in this investigation. Lines 10-15, 10-10, 5D3 and 5A4 were genotyped using primers that specifically amplify a region of the pBeloBAC vector sequence that is common to both the modified and unmodified transgene (Fig 2.2 A). *Phlda2* null animals were genotyped using primers that span the deleted region of *Phlda2*, thus producing wild type and KO products of distinct sizes (Fig 2.2 B).

From two weeks of age, mice were ear marked and up to 5 mm of tail removed under local anaesthetic. For embryonic stages, the yolk sac was separated from the embryo and placenta during dissection (Section 2.4) and used for genotyping. The tail biopsy or yolk sac was lysed in a shaking incubator at 55°C overnight in 500 μl of lysis buffer (50 mM Tris (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl and 0.5 % SDS in water) containing proteinase K (400 μg/ml). Tissue lysate was diluted 20-fold in 10 mM Tris (pH 8.0) and heated to 95°C for twenty minutes to denature proteinase K. Genotyping was performed in a total reaction volume of 25 μl (Table 2.4), using 1 μl of diluted lysate as template. Reactions were incubated at the relevant temperatures for the desired times in an MJ Research PTC-200 thermocycler.

	Volume (µl)
dH_2O	16.7
Buffer (10X) (Sigma)	2.5
$MgCl_2$ (25 mM) (Sigma)	2.0
dNTPs (10 mM each) (Roche)	0.8
Primers (Sigma Genosys)	1.0
Genomic Red Taq (Sigma)	1.0
Template DNA	1.0

Table 2.4: Reaction constituents for PCR genotyping

The table shows the volumes of each constituent required for a single PCR genotyping reaction. For multiple reactions a master mix was made.

BAC transgenic mice were genotyped using primers R85 and R86 (see Table 2.5 for sequences), designed using the Primer3 program (hosted at http://frodo.wi.mit.edu/primer3/input.htm). Thermocycler conditions were; 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, then 72°C for 3 minutes. Approximately half of each reaction was loaded on a 1% 1X Tris-acetate EDTA (TAE) agarose gel containing 0.5 µg/ml Ethidium Bromide (EtBr) (Sigma) (later SafeView (NBS Biologicals)) and visualised using a transilluminator (BioRad). Transgenic animals were identified by the presence of a ~575 bp product; with no product generated from wild type genomic DNA. Technical problems encountered with this genotyping protocol led to the design of a new set of primers. Primer set R85v2/R86v2 also amplifies a region of pBeloBAC vector sequence, with transgenic animals identified by the presence of a 314 bp product (Fig 2.2 C).

Genotyping of *Phlda2* null animals was performed using primers R293 and R294 (for sequences see Table 2.5). A touchdown PCR program was used in order to amplify both the 2,025 bp wild type and 479 bp KO products. Thermocycler conditions were; 94°C for 5 minutes, followed by 10 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 1 minute. A further 20 cycles were performed at 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute, then a final 72°C elongation step for 3 minutes. Primer sequences and thermocycler protocol were provided by Professor Ben Tycko. Products were resolved on a 1% TAE agarose gel as described above, with KO animals identified by the presence

of a ~500 bp product and wild type animals by a product of ~2-kb; the presence of both products identified heterozygous animals (Fig 2.2 D).

Primer	Sequence (5' – 3')	T_m (Primer3)
R85	ATT CTG CTT ACA CAC GAT GC	56.4
R86	TTC CGC AGA GGT CAA TCC	59.7
R85v2	GGG CAC CAA TAA CTG CCT TA	60.0
R86v2	GCG TGT TAC GGT GAA AAC CT	60.0
R293	CGA GAG CCT GCT TGG GAT TG	65.5
R294	GGG GCT TGT CCT CCA ATA AAA C	63.0

Table 2.5: Primer sequences for genotyping of transgenic mice

The table shows the sequences of primers used for genotyping transgenic lines. Primers R85 and R86 (later R85v2 and R86v2) were used for BAC-specific PCR, whereas R293 and R294 were used for *Phlda2* KO genotyping.

2.3.2 Genotyping by β-galactosidase staining

The β-galactosidase reporter gene is expressed from the modified transgene in neural tissue during embryonic development (John *et al.* 2001). This allows transgenic animals to be identified by the development of an intense blue stain in the neural cord and brain stem when embryos are incubated with the β-galactosidase substrate X-Gal (Promega). This method of genotyping was utilised to ascertain the genotype of embryos from lines 10-10 and 10-15 when the embryo was not required for further investigation.

Staining of embryonic stages E14.5 and earlier was performed on whole embryos. At developmental stages later than E14.5, embryos were decapitated according to Schedule 1, and development of colour assessed in the severed brain stem. Entire embryos or embryo heads were fixed in "*LacZ* fix" (2% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40, 1 mM $MgCl_2$ in phosphate buffered saline (PBS)) for one hour at 4°C on a rocking platform, washed in two changes of 1X PBS and then placed in "*LacZ* stain" (0.4 mg/ml X-Gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 1 mM $MgCl_2$, 0.02% NP-40 in PBS). Colour was allowed to develop in a lightproof box at room temperature for several hours. Transgenic conceptuses were identified by the development of an intense blue colouration; wild type embryos exhibit no such stain (Fig 2.2 E).

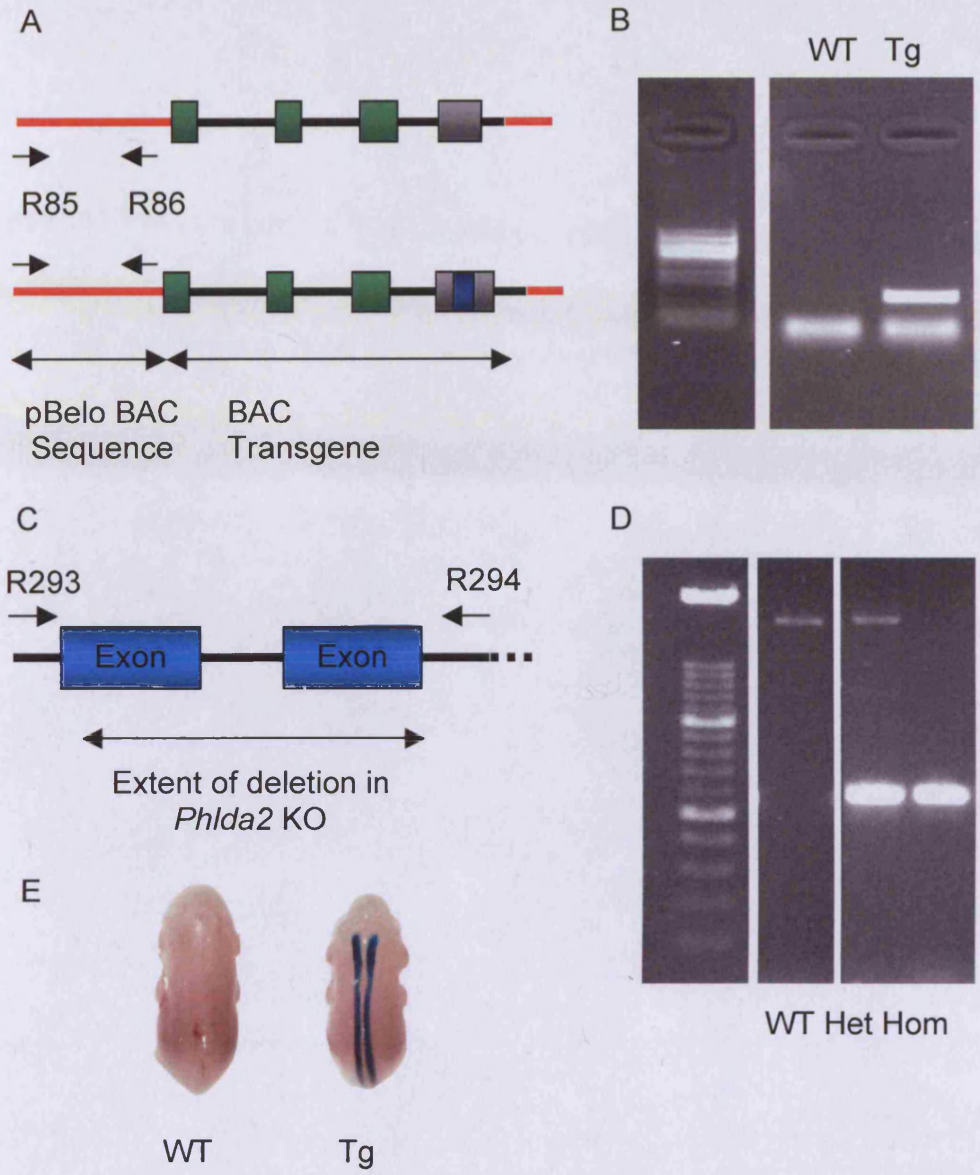


Figure 2.2: Genotyping of BAC transgenic and *Phlda2* KO mice

A: Position of primers R85 and R86 used for genotyping of lines 10-10. 10-15, 5D3 and 5A4, which amplify the same product from both unmodified (above) and modified (below) transgene. B: Result of genotyping reaction; transgenic animals were identified by a 314 bp (previously ~575 bp) product; wild type animals produce no product. C: Position of primers used to genotype *Phlda2* null KO mice. Primer R293 binds upstream of exon 1, with primer R294 binding downstream of exon 2. Both exons are excised in KO mice, however primer-binding sites remain intact. D: Result of genotyping reaction; wild type animals were identified by the presence of a 2025 bp product, *Phlda2* null animals were identified by the presence if a 479 bp product. The presence of both products indicated heterozygous animals. E: Development of characteristic blue colouration of neural tissue following *LacZ* staining of wild type and transgenic embryos from line 10-15 at E14.5.

2.4 *Dissection and fixation of embryos and placentae*

Transgenic males were mated with wild-type female 129/Sv mice, which were monitored for the presence of vaginal plugs, with the day of plug discovery recorded as day E0.5. At the required stages of gestation, pregnant females were killed by cervical dislocation, as per Schedule 1 and the uterus dissected out and placed into cold PBS. Embryos and placentae were dissected free of the uterus and placed separately into fresh, cold PBS in a 24-well plate. Yolk sacs were separated and placed into microfuge tubes, and stored at -20°C prior to lysis as described in Section 2.3.1. Embryos were killed by methods approved under Schedule 1 of the Animals (Scientific Procedures) Act 1986, including immediate decapitation or immersion in ice-cold fixative. Embryo and placental weights were ascertained after removing excess PBS/fixative by drying on a paper towel.

In order to preserve the integrity of tissue structure for histology and to prevent degradation of protein and nucleic acids, placentae were fixed in 4% paraformaldehyde (PFA) (4% PFA (Sigma) w/v in 1X PBS) at 4°C overnight. Fixative was removed and tissue washed three times for 10 minutes in cold PBS, once for 10 minutes in 50% EtOH and stored in 70% ethanol prior to embedding and sectioning. All solutions were made using diethyl pyrocarbonate (DEPC) treated sterile distilled water. In instances where embryos and/or placentae were required for subsequent extraction of RNA, tissue was immediately frozen in a 24-well plate placed directly onto dry ice, and stored at -80°C until required.

To assess relative organ size of transgenic animals, pups were dissected on the day of birth (P0). The entire litter was removed from the cage and maintained at a warm temperature in ample bedding under a lamp. Individual pups were weighed and decapitated as per Schedule 1. Brain, liver, heart, kidneys, lungs and pancreas were separated from the carcass and fixed in 4% PFA overnight at 4°C. Organs were subsequently washed in PBS and 50% EtOH as described for placentae above, and stored in 70% EtOH. After equilibrating in 70% EtOH for two days, organs were weighed and weights recorded.

2.5 *Embedding and sectioning of placentae*

Following fixation in PFA overnight, washing and transfer to 70% EtOH, placentae were processed, embedded and sectioned by the Histology Unit

(Cardiff School of Biosciences). Placentae were dehydrated for 1 hour sequentially in 70% EtOH, 95% EtOH, 100% EtOH (twice), xylene (twice), and passed through three changes of molten paraffin at 55°C for two hours each prior to being orientated and paraffin embedded. Midline sagittal sections of 10 μm thickness were cut and floated onto slides in a 37°C water bath. Slides were dried by incubating at 50°C overnight, and stored at room temperature in sealed slide boxes.

For *in situ* hybridisation, placentae were processed immediately following fixation. Placentae were dehydrated for two hours sequentially in 70% EtOH, 95% EtOH, 100% EtOH (twice), xylene (twice), and passed through three changes of molten paraffin at 55°C for two hours each prior to being orientated and paraffin embedded. Paraffin blocks were stored at 4°C in sealed containers prior to sectioning. Sections were prepared and stored at 4°C on the day prior to beginning the *in situ* protocol.

To measure the absolute volume of the spongiotrophoblast and labyrinth layers, placentae were PFA fixed as described previously and paraffin embedded by Histology Unit. Subsequently, sections of 10 μm thickness were cut throughout the entire depth of the placenta using a Microm HM310 microtome. The first and every tenth section thereafter was retained and placed onto slides in a known order prior to H&E staining. Cross-sectional area was measured as described in Section 2.10.

2.6 Haematoxylin and eosin staining

Haematoxylin and Eosin (H&E) staining of placental midline sections was used to assess placental structure and determine the relative contribution of spongiotrophoblast and labyrinth regions. The protocol used for H&E staining was provided by Katrina Varanou from the University of Cambridge.

Slides were deparaffinised in two changes of xylene for 10 minutes, and rehydrated through graded ethanol solutions for 2 minutes each; 100% (twice), 95%, 70%, 50%, and 30% EtOH, followed by five minutes in dH_2O. Slides were immersed in Mayer's haematoxylin (Sigma) for two minutes and washed in running tap water for 5 minutes. Slides were dipped in acid alcohol solution (0.5% HCl in 70% EtOH) for 10 seconds, and rinsed again in running tap water for 5 minutes. Acid alcohol treatment was repeated, and slides washed first in running

tap water, then in dH_2O for five minutes each. Slides were immersed in Eosin Y Solution (Sigma) for 5 - 10 seconds and immediately dehydrated through graded ethanol solutions of 30%, 50%, 70%, and 95% each for 5 - 10 seconds, then 100% EtOH for 2 minutes. Slides were fixed in xylene for 5 minutes and mounted using DPX mounting medium (Sigma).

2.7 Detection of glycogen cells

Glycogen cells are a unique subtype of trophoblast found in the murine placenta, characterised by large glycogen-filled vacuoles (Georgiades *et al.* 2002). Two main techniques have been previously used to identify these cells, the first to be used was Best's Carmine staining, which results in the staining of glycogen a bright pink colour, with some mucins also staining a less intense colour (Lopez *et al.* 1996). More recently, periodic acid Schiff (PAS) staining has been widely used to identify such cells (including Bouillot *et al.* 2005; Coan *et al.* 2006). Both techniques were employed in this investigation in order to compare the relative effectiveness of each technique, and to assess the relative extent of glycogen staining in wild type and transgenic placentae.

In addition to staining for glycogen in PFA fixed placentae, the use of an alternative method of fixation was also investigated. Placentae were dissected as described previously and placed directly into Tellyesniczky/Fekete (Telly's) fixative (20 parts 70% EtOH, 2 parts 40% Formalin, 1 part glacial acetic acid), as recommended by the Jackson Laboratory for the preservation of glycogen (http://jaxservices.jax.org/histology-fixatives.html). Following overnight fixation at 4°C, placentae were washed and stored in 85% EtOH prior to processing and embedding as described for PFA fixed placentae, but omitting the dehydration step through 70% EtOH. Midline sections of 10 μm thickness were cut and floated onto slides as described in Section 2.5.

2.7.1 Periodic acid Schiff staining

Periodic acid Schiff (PAS) staining is based upon two chemical reactions, the first is an oxidation of glycogen by periodic acid to form aldehyde groups, and the second involves the reaction of these aldehyde groups with Schiff's reagent to develop an intense pink colouration.

Slides were deparaffinised as described in section 2.6, and rehydrated through 100% (twice), 95% and 70% EtOH. Sections were circled using a PAP-pen (Dako), and incubated with 1 ml of 1% (w/v) periodic acid (Sigma) in dH_2O for 5 minutes at room temperature. Slides were washed in running water for 5 minutes and incubated in 1 ml Schiff's reagent (Sigma) for 10 minutes at room temperature. Slides were rinsed in running tap water for a further 10 minutes, counterstained with haematoxylin for 1 minute and excess stain cleared by washing in tap water. Slides were dehydrated by reversing the rehydration steps and mounted using DPX mounting medium.

2.7.2 Best's Carmine staining

Slides were deparaffinised and rehydrated to water as described in section 2.6. Sections were stained in Mayer's haematoxylin (Sigma) for two minutes and washed in running tap water for 5 minutes. Slides were dipped in acid alcohol solution (0.5% HCl in 70% EtOH) for up to 10 seconds, and rinsed briefly in running tap water. Slides were stained in Best's Carmine solution (Sigma) for 6 minutes, washed in Best's differentiator (40% EtOH, 20% MeOH in dH_2O) for 2 minutes and 100% EtOH for 2 minutes. Slides were cleared in xylene for 5 minutes and mounted using DPX medium.

2.7.3 Comparison of glycogen staining techniques

A comparison of the four possible combinations for fixation and glycogen staining was performed in order to determine the method that would yield the best results. Initial observations suggested that Best's Carmine staining of PFA fixed placental sections yielded appropriate staining with good distinction between glycogen and other cell types (Fig 2.3A). However, PAS staining is more extensively utilised in recent publications, and yielded good results with both PFA-fixed sections and Telly's fixed sections, resulting in intense pink staining of glycogen cells (Fig 2.3B and D respectively). Best's Carmine staining displayed poor reproducibility, with colour not developing for either PFA or Telly's fixed placentae in some instances (Fig 2.3C and E respectively). PAS staining of PFA-fixed tissue was subsequently used to detect glycogen cells because of the ability to use PFA-fixed placentae for other histological techniques, including *in situ* hybridisation and the reproducibility of PAS staining over Best's Carmine.

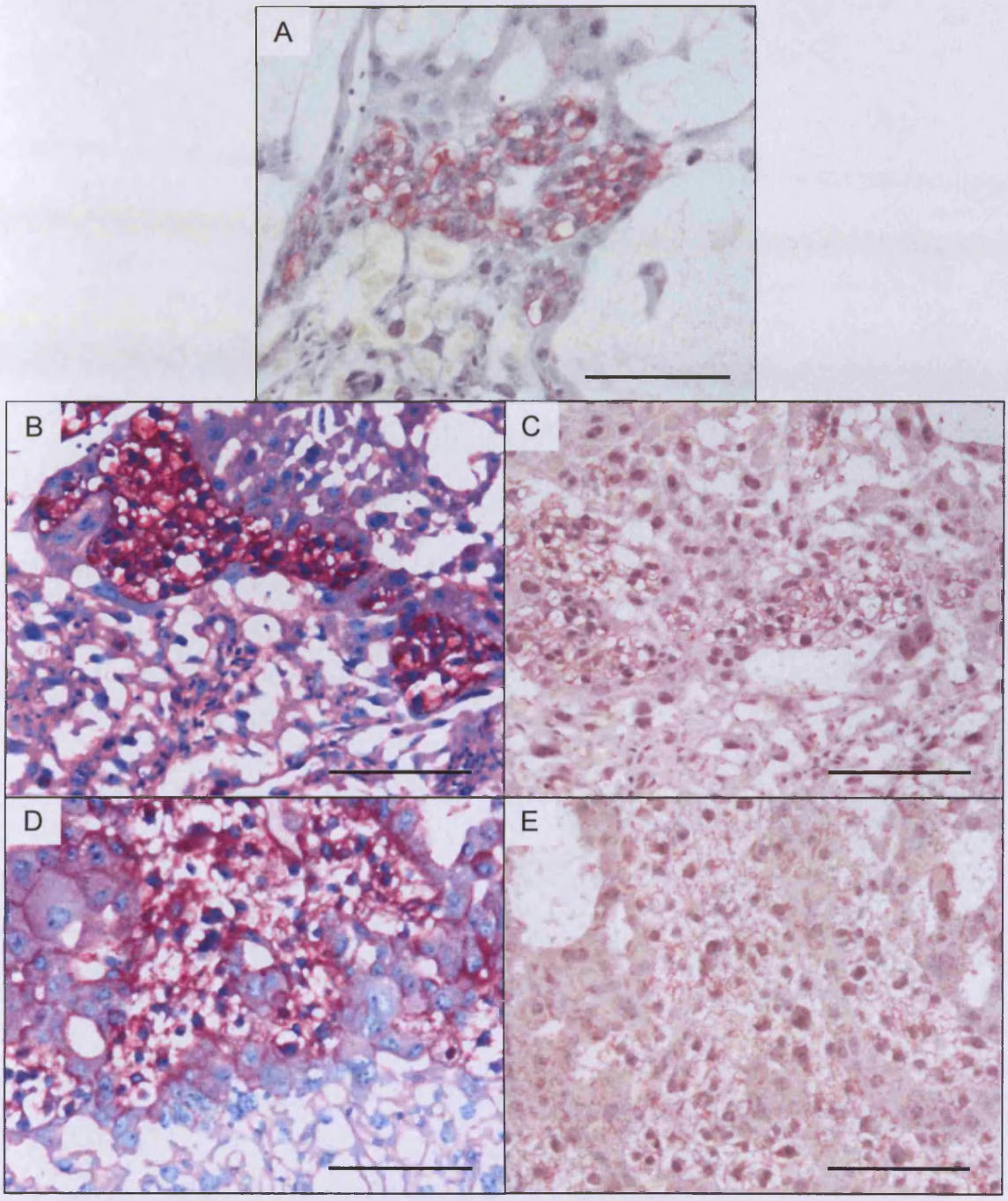


Figure 2.3: Comparison of glycogen staining techniques

Fixation and staining method combinations were compared to determine the optimum conditions for visualisation of glycogen cells. A: Best's Carmine staining of a PFA fixed section. B and C: PAS and Best's Carmine staining respectively of adjacent PFA-fixed sections. D and E: PAS and Best's Carmine staining respectively of adjacent PFA-fixed sections. Scale bar = 100 μm.

2.8 *In situ hybridisation*

In situ hybridisation was used to examine the spatial and temporal expression of mRNA for a number of key placental markers. The technique utilises digoxigenin (DIG) labelled RNA probes (riboprobes) that are complementary (anti-sense) to and thus specifically bind the target mRNA sequence. An anti-digoxigenin alkaline phosphatase-conjugated antibody was subsequently used to detect probe binding by development of a purple stain. DIG labelled sense RNA probes of the same sequence as the mRNA molecules were used as a negative control. Probes were transcribed from a vector containing a full-length or partial cDNA fragment of the appropriate mRNA in the multiple cloning site (MCS), flanked on each side by a promoter sequence for T3, T7 or SP6 RNA polymerase (Fig 2.4 A). The RNA polymerase promoters were used to sequence the cDNA insert in order to ascertain its orientation within the vector. The vector was then linearised by two separate restriction enzymes, one cutting on each side of the insert, such that the appropriate RNA polymerase could be used to transcribe "run-off" probes of consistent length (Fig 2.3 B).

Riboprobes for *Phlda2* and *Slc22a18* were designed and generated specifically for this investigation. In addition to these, a number of previously published probes were also utilised to assess the relative expression of various placental markers. The junctional zone marker *Tpbpa* is only expressed by spongiotrophoblast and glycogen cells (Lescisin *et al.* 1988). *Ascl2* is expressed in the pre-implantation embryo, becoming restricted to the trophoblast lineage at implantation. High levels of expression are observed in the ectoplacental cone and chorionic epithelium at E7.5, with stronger expression seen in the ectoplacental cone at E8.5. From ~E10.5, expression patterns in both spongiotrophoblast and labyrinth regions becomes patchy and begins to decline, with little *Ascl2* expression at E18.5 (Guillemot *et al.* 1994; Rossant *et al.* 1998). *Cdkn1c* mRNA is detected in cells of both the spongiotrophoblast and labyrinth layers, but was not detected in glycogen cells or giant cells (Takahashi *et al.* 2000). In contrast, others have found high levels of *Cdkn1c* protein localised to glycogen cells (Coan *et al.* 2006; Georgiades *et al.* 2001). Labyrinth development was examined by assessment of the expression pattern of *Gcm1*, which is expressed at the branch-points of the dividing epithelium (Altshuller *et al.* 1996;

Basyuk *et al.* 1999). *Hand1* is expressed in cells of the spongiotrophoblast and labyrinth, and acts to promote giant cell differentiation in opposition to the effect of *Ascl2* (Cross *et al.* 1995; Riley *et al.* 1998; Scott *et al.* 2000). During early gestation, giant cells were examined by expression of prolactin family 3, subfamily d, member 1 (*Prl3d1*, formerly placental lactogen 1 (*Pl1*)), which is expressed from at least E6.5 until approximately E11.5 (Carney *et al.* 1993; Simmons *et al.* 2008; Yamaguchi *et al.* 1992). From E12.5, giant cells were examined by expression of prolactin family 3, subfamily b, member 1 (*Prl3b1*, formerly placental lactogen 2 (*Pl2*)), which is expressed in all giant cell sub-types except the spiral artery associated giant cells in addition to the spongiotrophoblast cells (Simmons *et al.* 2008). Fetal capillaries in the labyrinth were detected by expression of the VEGF receptor *Kdr* (*Flk1*) (Breier *et al.* 1995; Dumont *et al.* 1995).

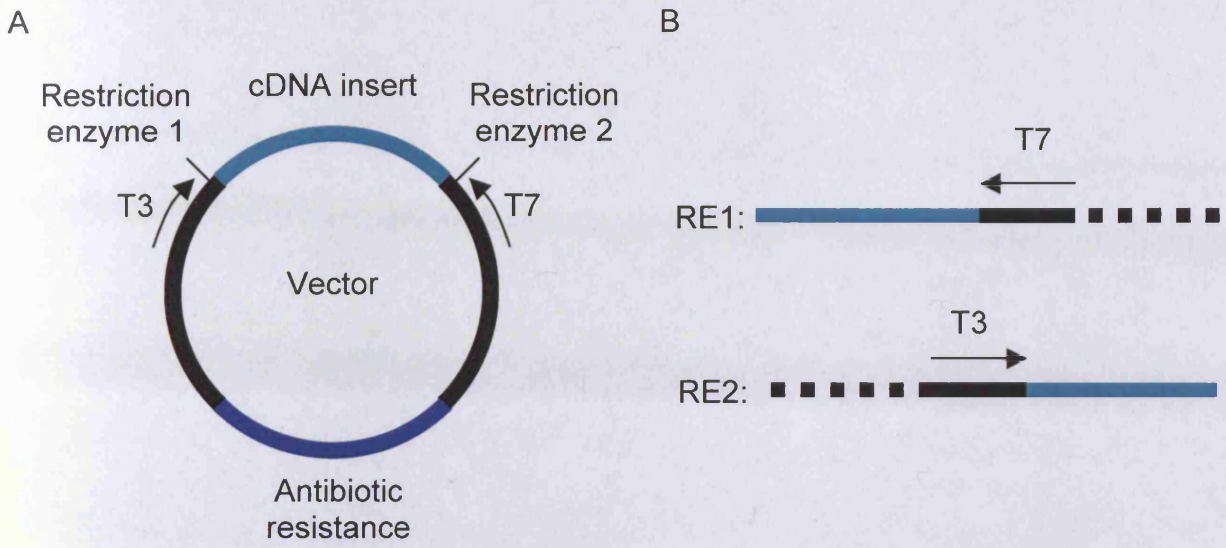


Figure 2.4: Schematic of in situ "run-off" probe transcription

Sense and antisense run-off riboprobes were transcribed from a linearised plasmid construct containing an appropriate cDNA. A) Full length or partial cDNA fragments were cloned into appropriate vectors with an MCS flanked by RNA polymerase promoter sequences (T3 and T7 or T7 and SP6). The orientation of the cDNA was determined by sequencing using promoters specific to the RNA polymerase promoter sites. The primer that resulted in a complementary sequence to the mRNA transcript is the anti-sense probe. B) Plasmid DNA was linearised in two separate reactions on each side of the cDNA insert, and run-off riboprobes were transcribed with the appropriate RNA polymerase. In this example the cDNA fragment was cloned into a vector containing promoters for the RNA polymerases T3 and T7. Probe was transcribed using T7 RNA polymerase from plasmid linearised with restriction enzyme 1, and using T3 RNA polymerase from plasmid linearised with restriction enzyme 2.

2.8.1 Generation of *Phlda2* and *Slc22a8* cDNA constructs

Primer sets R356/357 and R358/359 were designed using Primer3 to amplify fragments of the *Phlda2* and *Slc22a18* coding regions respectively (see Table 2.6 for sequences). Primer set R356/357 amplifies a 546 bp region of the *Phlda2* spliced transcript, between nucleotides 120 and 666 inclusive (NCBI accession number NM009434). Primer set R358/359 amplifies an 858 bp region of the *Slc22a18* spliced transcript, between nucleotides 440 and 1297 inclusive (NCBI accession number NM008767).

Primer	Sequence (5' – 3')	T_m (Primer3)
R356	ACA GCC TGT TCC AGG TAT GG	60.0
R357	GGT TGG AAG CAG GTA ACC AA	60.0
R358	AGA GCG GCA CTC TCA CTC TC	60.0
R359	GGT CAA TGG TTG CAC AGA TG	60.0

Table 2.6: Primer sequences for amplification of *Phlda2* and *Slc22a18* cDNA

Primer3 was used to design primers to amplify fragments of *Phlda2* and *Slc22a18* transcripts in order to generate riboprobes for *in situ* hybridisation.

Target sequences were amplified from wild type E12.5 placental cDNA (see Sections 2.11.1 and 2.11.2 for generation of cDNA) in separate reactions. For each target, four separate reactions were set up according to Table 2.7, using 2 μl of diluted cDNA as template. Thermocycler conditions were 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, then a 72°C final elongation step for 3 minutes. For each target, the four reactions were combined in a single microfuge tube and 10 μl analysed on a 1.5% 1X TAE gel, using a 100 bp ladder (NEB) to quantify product size. PCR-amplified products were purified using a PCR purification kit (Qiagen) according to the manufacturer's instructions and resuspended in 50 μl 10 mM Tris (pH8.0). Concentration was determined from 1 μl using a NanoDrop 1000 (Thermo Scientific) and DNA stored at -20°C.

	Volume (μl)
dH_2O	33.4
Buffer (10X) (Sigma)	5.0
$MgCl_2$ (25 mM) (Sigma)	4.0
dNTPs (10 mM each) (Roche)	1.6
Primers (Sigma Genosys)	2.0
Genomic Red Taq (Sigma)	2.0
Template DNA	2.0

Table 2.7: Reaction constituents for PCR amplification of *Phlda2* and *Slc22a18* cDNA

The table shows the constituent volumes for PCR amplification of cDNA fragments of *Phlda2* and *Slc22a18*. Gene fragments were amplified in four separate reactions each containing the above constituents.

Amplified cDNA fragments were ligated into the pGEM-T Easy Vector System (Promega). The pGEM-T Easy Vector contains a single 3' deoxythymidine overhang, which is complementary to the additional deoxyadenosine that is added to the 3' end of PCR products by most *Taq* polymerase enzymes. Two separate ligation reactions were set up for each target, with either a 3:1 or a 5:1 insert to vector molar ratio, calculated using the formula below:

$$\text{Amount of insert (ng)} = \frac{\text{amount of vector (ng) x insert size (kb)}}{\text{vector size (ng)}} \times \frac{\text{insert}}{\text{vector}} \text{ ratio}$$

Ligation reactions were incubated overnight at 4°C. Following brief centrifugation, 2 μl of ligation mixture was mixed with 50 μl of high efficiency JM109 cells (Promega) in a 1.5 ml microfuge tube. Cells were incubated on ice for 20 minutes, heat shocked at 42°C for 45 seconds to facilitate uptake of plasmid and immediately placed on ice for 2 minutes. Room temperature SOC medium (950 μl) was added to each tube and incubated at 37°C in a shaking incubator for 90 minutes. Each culture was plated onto two agar plates containing carbenicillin (Sigma), IPTG (Sigma) and X-Gal (Promega). One plate was inoculated with 100 μl and the other with 900 μl of culture, and incubated at 37°C overnight. The pGEM-T Easy Vector contains a *β-galactosidase* reporter gene, which is inactivated following successful ligation of a DNA fragment into the

vector, thus allowing positive transformants (white colonies) to be selected over negative colonies (blue).

Five white colonies were selected from each of the 100 μl plates and used to inoculate 5 ml of LB medium containing carbenicillin, which was incubated overnight at 37°C in a shaking incubator. Plasmid DNA was purified from 4.5 ml of each culture using a mini prep kit (Qiagen), according to the manufacturer's instructions, with DNA eluted in 50 μl dH_2O. The remaining 0.5 ml of each culture was used to generate a "glycerol stock" by mixing with an equal volume of 50% glycerol in a sterile screw cap tube, labelled appropriately and stored at -80°C. Plasmid DNA was quantified using a NanoDrop, and an *Eco*RI analytical digest, designed to excise the cloned fragment, performed on approximately 500 ng of DNA from each culture. DNA from cultures exhibiting the expected fragment size was sent for sequencing at the Cardiff Sequencing Core utilising the SP6 and T7 promoter sites that flank the multiple cloning site (MCS) of the pGEM-T Easy Vector. Insert sequences were aligned with published mRNA sequences available from NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene). Insert orientation with respect to SP6 and T7 promoters was determined and any mismatches present in the cloned sequence were noted. Only constructs with sequences identical to the published mRNA sequence were retained for future use. Glycerol stocks of transformants that did not display 100% sequence identity were discarded, whereas glycerol stocks of those colonies that met this criterion were stored at -80°C until required, with riboprobes prepared as described in Sections 2.8.3 and 2.8.4.

2.8.2 Transformation of competent cells with cDNA vectors

In addition to the constructs created for *Phlda2* and *Slc22a18* riboprobes, several previously published *in situ* probes were also utilised. Constructs for these probes were supplied as either a small volume (~5 μl) of purified plasmid DNA in Tris, or "spotted" on a piece of 3MM paper, outlined in pencil and sealed in a sterile bag. Plasmids that were supplied in Tris were stored at -20°C until required, whereas those that were spotted on 3MM paper were placed in a sterile microfuge tube, covered with the minimum amount of 10 mM Tris (pH 8.0), incubated at room temperature for 5 minutes, and stored at -20°C.

In order to facilitate large-scale purification of plasmid DNA, each construct was transfected into chemically competent JM109 *Escherichia coli* (Promega), which were subsequently cultured and plasmid DNA extracted by midi prep. Competent cells (50 μl) were mixed with ~1 μg plasmid DNA (approximately 1-2 μl) and incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 45 seconds and placed on ice for 2 minutes. Room temperature SOC medium (950 μl) was added and the culture incubated for 90 minutes at 37°C in a shaking incubator. Cultures were plated onto two agar plates containing the relevant antibiotic as described in Section 2.8.1, and plates incubated at 37°C overnight.

A single colony from the 100 μl plate was used to streak a second agar plate containing antibiotic and incubated overnight at 37°C. Four well-isolated individual colonies were selected from the second plate and grown overnight in a shaking incubator at 37°C in 5 ml of LB medium containing the appropriate antibiotic. Plasmid DNA was isolated from 4.5 ml of culture using a mini prep kit (Qiagen), following the manufacturers instructions and DNA eluted in 50 μl sterile H_2O. A glycerol stock for each culture was prepared as described in Section 2.8.1 and stored at -80°C.

In order to confirm that the correct construct had been transfected and purified, an appropriate analytical digest was performed on approximately 500 ng of plasmid DNA. Each analytical digest was designed to excise the cloned insert, thus generating two products, representing the vector backbone and the insert. Table 2.8 shows the expected size of the cDNA fragment for each construct when digested with the restriction enzyme shown. Restriction enzyme digests were performed in reaction volumes of 10 μl (Table 2.9) and incubated at the appropriate temperature for 4 hours prior to being analysed on a 1.5% TAE agarose gel. One sample exhibiting the correct insert size was selected for each construct and sequenced to ensure the correct cDNA fragment had been inserted and to confirm its orientation with respect to the RNA Polymerase promoters. Sequencing was performed from both promoters using 300 ng of DNA per reaction and carried out at the Cardiff Sequencing Core, and sequences compared with published mRNA sequences available from NCBI.

Target	Vector	Resistance	Analytical Digest	Fragment Size
Ascl2	pBlueScript	Ampicillin	*Eco*RI	1,700 bp
Cdkn1c	pBlueScript	Ampicillin	*Xho*I	1,400 bp
Kdr	pCDNA3	Ampicillin	*Eco*RI	1,500 bp
Gcm1	TopoII	Ampicillin	*Eco*RI	400 bp
Hand1	pBlueScript	Ampicillin	*Sal*I and *Not*I	1,700 bp
Phlda2	pGEM T-Easy	Ampicillin	*Eco*RI	550 bp
Pl1	pGEM2	Ampicillin	*Hind*III and *Pst*I	800 bp
Pl2	pGEM1	Ampicillin	*Hind*III and *Sac*I	800 bp
Slc22a18	pGEM T-Easy	Ampicillin	*Eco*RI	850 bp
Tpbpa	pBlueScript	Ampicillin	*Eco*RI	750 bp

Table 2.8: Constructs used for generation of riboprobes

The table depicts the vector, antibiotic resistance, restriction enzyme used for analytical digest and size of insert for each riboprobes construct. All restriction enzymes were from New England Biolabs (NEB).

	Volume (µl)
H_2O	to 10
Buffer (10X)	1.0
BSA (100X)	0.1
Enzyme	1 (0.5 each for double digest)
Plasmid DNA	0.5 - 1 µg

Table 2.9: Reaction constituents for analytical digests

Analytical restriction enzyme digests were performed to confirm the isolation of the anticipated construct. The table shows the constituent volumes for a single restriction enzyme digest reaction of mini or midi prep plasmid DNA.

2.8.3 Midi prep of plasmid DNA and linearisation of probe constructs

Agar plates containing the appropriate antibiotic were inoculated using the glycerol stock of the required construct and incubated overnight at 37°C. A single, well-isolated colony was used to inoculate a second agar plate, and incubated overnight at 37°C. A single colony from the second agar plate was used to inoculate 5 ml of LB medium containing antibiotic and grown for 8 hours at 37°C in a shaking incubator at 200 rpm. 100 μl of this culture was used to inoculate 200 ml LB medium, from which plasmid DNA was purified using a midi prep kit (Qiagen) following the manufacturer's instructions, with DNA resuspended in 200 μl 10 mM Tris (pH 8.0), and stored at -20°C. Analytical digests were performed on plasmid DNA preps as described in Section 2.8.2.

Two separate reactions were set up using the restriction enzymes shown in Table 2.10 to linearise each construct either side of the insert thus allowing the independent transcription of sense and anti-sense probes. A total of 30 μg of DNA was linearised in each reaction, with constituents as shown in Table 2.11. Restriction digests were incubated overnight at the appropriate temperature, and 5 μl of reaction was run on an agarose gel alongside undigested vector to confirm complete linearisation.

Linearised plasmid DNA was phenol/chloroform extracted by first adjusting the volume to 500 μl with 10 mM Tris (pH 8.0), then adding an equal volume (500 μl) of phenol and mixing. The mixture was centrifuged at 13,000 rpm for 10 minutes at room temperature, and the upper aqueous phase was removed to a clean microfuge tube to which an equal volume of chloroform was added and mixed. The mixture was centrifuged as above, and the aqueous phase removed to another microfuge tube. A volume of 3 M NaOAc (pH 5.2) equal to one-tenth of the total volume of the aqueous phase was added in addition to 2.5 volumes of 100% EtOH. DNA was precipitated overnight at -20°C, and centrifuged for 20 minutes at 13,000 rpm at 4°C. The pellet was washed with 1 ml of 70% EtOH, allowed to air dry and resuspended in 25 μl of 10 mM Tris (pH 8.0). DNA concentration was determined using a NanoDrop as before, and adjusted to 1 μg/μl using 10 mM Tris (pH 8.0) as required.

	Anti-sense probe	Sense probe
Ascl2	*Sma*I	*Cla*I
Cdkn1c	*Sac*I	*Kpn*I[1]
Kdr	*Cla*I	*Bam*HI
Gcm1	*Hind*III	*Xho*I
Hand1	*Sal*I	*Not*I
Phlda2	*Pst*I	*Sac*II
Pl1	*Hind*III	*Pst*I
Pl2	*Hind*III	*Sac*I
Slc22a18	*Pst*I	*Sac*II
Tpbpa	*Xba*I	*Kpn*I[1]

Table 2.10: Restriction enzymes for plasmid linearisation

Constructs were linearised in two separate reactions using the restriction enzymes stated above. All restriction enzymes were obtained from NEB except [1] from Promega.

	Volume
RNase Free H_2O	to 200 μl
Buffer (10X) (NEB)	20 μl
BSA (1X) (NEB)	1 μl
Enzyme	10 μl
DNA	30 μg

Table 2.11: Reaction constituents for linearisation

The table shows the constituent volumes for a single reaction to linearise a construct containing a cDNA fragment from which run-off DIG labelled riboprobes were generated.

2.8.4 DIG labelling of riboprobes

The linearised plasmid DNA prepared in Section 2.8.3 was used as a template for the transcription of Digoxigenin-labelled riboprobes using SP6, T3 or T7 RNA polymerase (Roche) as detailed in Table 2.12. Reaction constituents for DIG-labelling of riboprobes are shown in Table 2.13.

	Anti-sense probe	Sense probe
Ascl2	T3	T7
Cdkn1c	T3	T7
Kdr	T7	T3
Gcm1	T7	SP6
Hand1	T7	T3
Phlda2	T7	SP6
Pl1	T7	SP6
Pl2	T7	SP6
Slc22a18	T7	SP6
Tpbpa	T3	T7

Table 2.12: RNA Polymerase enzymes for probe transcription

Run-off DIG-labelled riboprobes were transcribed from linearised plasmid DNA using the appropriate RNA polymerase enzyme from Roche.

	Volume
RNase Free H_2O	12 μl
Transcription Buffer (10X) (Roche)	2 μl
DIG RNA Labelling mix (Roche)	2 μl
RNasin (Promega)	1 μl
RNA Polymerase (Roche)	2 μl
DNA	1 μl (1 μg/μl)

Table 2.13: Reaction constituents for DIG labelling

The table shows the constituent volumes for a single reaction for the generation of a DIG labelled run-off riboprobe.

The DIG RNA labelling mix contains the nucleotide triphosphates ATP, CTP and GTP at a concentration of 10 mM. UTP is present at a concentration of 6.5 mM and is supplemented with 3.5 mM DIG-11-UTP, to make a total concentration of 10 mM. In this way, as the riboprobes are transcribed, approximately one in every three UTP molecules contains the DIG label.

Transcription reactions were incubated for 2 hours at 37°C, and DNA template digested by incubation with 20 units of DNaseI (Ambion) for 15 minutes at 37°C. Probes were precipitated by addition of 2 μl 3 M NaOAc (pH 5.2) and 100 μl 100% EtOH, and incubated at -20°C overnight. RNA was pelleted by centrifugation at 13,000 rpm at 4°C for 20 minutes, the pellet washed with 70% EtOH, and allowed to air dry for a few minutes. The pellet was resuspended in 100 μl of DEPC treated H_2O and divided into 10 μl aliquots, which were stored at -80°C.

2.8.5 Probe hybridisation

Slides were dewaxed in two changes of xylene for 10 minutes each, and rehydrated through graded ethanols of 100% (twice), 95%, 85%, 75%, 50% and 30% for 1 minute each. Slides were incubated in 1X saline followed by 1X PBS for 5 minutes each and incubated in 6% hydrogen peroxide (in 1X PBS) for 30 minutes at room temperature in order to inactivate endogenous alkaline phosphatase activity. Slides were washed twice in 1X PBS for 5 minutes each, fixed in ice cold 4% PFA (in 1X PBS) for 20 minutes and washed twice in 1X PBS for 5 minutes each. Slides were incubated for 5 minutes in Proteinase K (Melfords) (20 μg/ml Proteinase K, 50 mM Tris, 5 mM EDTA in H_2O) at room temperature, washed in 1X PBS for 5 minutes, post-fixed in 4% PFA for 5 minutes and washed in DEPC treated H_2O for 2 minutes.

To reduce non-specific probe binding, slides were treated with acetic anhydride (0.01 M in 0.1 M triethanolamine hydrochloride), by incubating at room temperature for 10 minutes. Slides were washed with 1X PBS and 1X saline each for five minutes at room temperature. Slides were rehydrated through 30%, 50%, 70%, 85%, 95% and 100% (twice) EtOH solutions, each for 1 minute, except for 70%, in which slides were left for five minutes to prevent salt precipitation. Slides were air dried for 30 minutes, and placed into sealable boxes lined with absorbent paper soaked in moisture buffer (5X SSC, 50% (v/v) formamide in

DEPC H_2O). Probe was heated at 80°C for 3 minutes, and stored on ice, whilst hybridisation buffer (5X SSC, 50% formamide, 1% SDS, 0.05 mg/ml heparin, 0.05 mg/ml calf liver tRNA in DEPC H_2O) was thawed at 80°C. An appropriate amount of probe and hybridisation buffer were mixed (per slide: 1 μl probe per 100 μl hybridisation buffer), and applied to relevant slides. A piece of parafilm was placed on top of the probe mixture, the box sealed with electrical tape and incubated at 62°C overnight in a water bath.

2.8.6 Post-hybridisation treatment

Slides were incubated in pre-warmed 5X SSC at 65°C for 30 minutes, and parafilm removed from solution using forceps. Slides were washed twice in pre-warmed solution I (50% formamide, 5X SSC, 1% (v/v) SDS in DEPC H_2O) at 65°C for 30 minutes each, and then washed three times in solution II (0.5 M NaCl, 0.01 Tris (pH 7.5), 0.1% Tween 20 in DEPC H_2O) at room temperature for 10 minutes each. Slides were treated with RNase (0.02 mg/ml in solution II) to digest unhybridised probe, by incubation at 37°C for 45 minutes, followed by a final 10 minute was in solution II at room temperature. Slides were washed twice at 65°C in solution III (50% formamide, 5X SSC in DEPC H_2O) for 30 minutes each. Slides were washed twice in PBT (0.1% Tween 20 in 1X PBS) for 10 minutes each and pre-blocked in 10% heat-inactivated sheep serum in PBT for 3 hours, covered with a piece of parafilm as before. Anti-digoxigenin alkaline phosphatase conjugated antibody (Roche) was pre-absorbed for 3 hours at 4°C in 1% heat inactivated sheep serum in PBT containing 6 mg/ml dried, homogenised wild type placenta. Parafilm was removed from slides by washing in PBT for 5 minutes, and the pre-absorbed antibody diluted to a final concentration of 1:2000 in 1% sheep serum in PBT. Antibody solution (100 μl) was applied to each slide, covered with a piece of parafilm, and incubated overnight at 4°C.

2.8.7 Signal detection

Parafilm was removed from slides by washing in PBT for 5 minutes, followed by a further two five minute washes in PBT, and a subsequent three washes for 30 minutes in PBT. Slides were preconditioned in three washes of NTMT (0.1 M NaCl, 0.1 M Tris (pH 9.5), 0.05 M $MgCl_2$, 0.1% Tween 20) containing 2 mM

Levamisole (Sigma) for five minutes each. BM Purple substrate (Roche) was applied directly onto each slide to completely cover sections. Slides were sealed in a dark box and incubated at room temperature until signal of the desired intensity developed. Slides were washed in PBT for 10 minutes, dH_2O for 30 minutes, and counterstained in Eosin Y Solution (Sigma) for up to 10 seconds. Excess eosin was washed in running tap water and slides air dried for 30 minutes. Slides were cleared briefly in xylene, air dried and mounted in DPX mounting medium (Sigma).

2.8.8 Optimisation of *in situ* hybridisation

A number of steps were optimised during the development of the *in situ* hybridisation protocol. An important consideration was to prevent the slides from drying out following application of the probe. For this reason, slides were incubated in a water bath during the probe hybridisation step. Initially this step was performed in an incubator, which resulted in slides drying out and poor signal intensity after staining. Similarly, parafilm was used to prevent the slides drying out during hybridisation, pre-blocking and incubation with secondary antibody. Initially parafilm was only used during the hybridisation step, with the secondary antibody solution tending to drain from the slides during the overnight incubation.

Following these adjustments to the protocol the signal intensity and section integrity were determined to be suitable for experimental use. A number of slides were subjected to the *in situ* protocol using various probes. These slides were mounted using VectaMount Permanent Mounting Medium (Vector Laboratories). However, after several months the majority of slides developed a precipitate in the mounting medium that occasionally obscured the section. Furthermore, the intensity of the eosin counterstain had dramatically reduced. Subsequently all slides that were subjected to *in situ* hybridisation were mounted using DPX mounting medium and to date no such precipitate or de-staining has been observed. Due to the large number of slides that had been subjected to *in situ* hybridisation at this stage it was not feasible to repeat all experiments. In most instances a representative section could be found that was not obscured by the precipitate, and which demonstrated the pattern of staining appropriately. A comparison of the effects of the two mounting media used is shown in Figure 2.5.

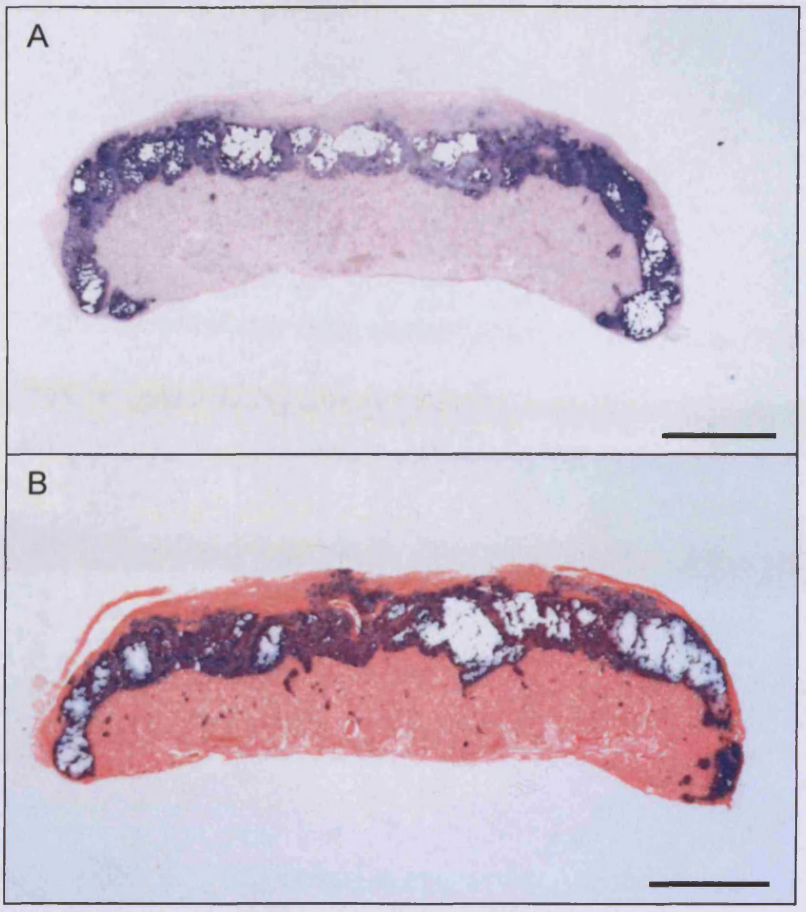


Figure 2.5: Effect of mounting medium on *in situ* hybridised section integrity

Two mounting media were used for *in situ* hybridisation. A) Slides that were hybridised with riboprobes were initially mounted with VectaMount, which resulted in the development of a precipitate in the mounting medium of some sections (not shown) and decolouration of eosin staining. B) Subsequent slides were mounted in DPX mounting medium, which preserved the intensity of eosin staining and did not result in any precipitate formation. Scale bar = 1 mm.

2.9 Digital photographs of placental sections

Photographs of placental sections were taken at varying degrees of magnification both for visualisation and presentation of the placental phenotype and in order to quantify the relative proportion of spongiotrophoblast and labyrinth regions. Photographs of entire placental midline sections from which spongiotrophoblast and labyrinth regions were measured were taken under low magnification using a Nikon SMZ1000 dissection microscope mounted with a Nikon CoolPix digital camera. Presentation quality photographs of all tissues including placentae were taken under various degrees of magnification between 2X and 20X using an Olympus BX41 light microscope mounted with a ColorView III Soft Imaging System digital camera operated through analySIS computer software.

2.10 Measurement of placental areas and volumes

Spongiotrophoblast and labyrinth areas were directly measured from H&E stained placental sections (Section 2.6) using the ImageJ freeware developed by NIH and downloaded from http://rsb.info.nih.gov/ij. Calibration was achieved by measuring a known distance on a photograph of a ruler and setting the measured length in pixels to equal the known distance. Photographs taken as described in Section 2.9 were opened in ImageJ and the "Freehand Selection" tool used to outline the relevant regions and derive their respective area. Spongiotrophoblast and labyrinth regions were measured both separately and together (total area).

Spongiotrophoblast and labyrinth volumes were measured from E14.5 placenta. Whole placentae were sectioned end-to-end as described in Section 2.5, photographed as in Section 2.9 and areas measured from each section as described above. Each area measured corresponded to every tenth section throughout the placenta, at a known distance of 100 μm apart. The measured areas together with the distance between sequential sections allowed the volume to be determined by the trapezium rule, using the formula below:

$$\text{Volume } (mm^3) \approx 50 \left(A_0 + A_n + 2 (\Sigma A_r) \right)$$

Where A_0 is the area of the first section; A_n is the area of the final section; and A_r represents the areas of all sections in between.

2.11 Analysis of gene expression by qPCR

Expression of *Phlda2*, *Slc22a18* and *Cdkn1c* was assessed in placentae of lines 10-15, 10-10 and 5D3 at E12.5 and E16.5. The relative expression of other genes of interest in the placenta, including nutrient transporters, was also assessed at E16.5 in lines 10-15 and 5D3. Quantitative reverse transcription polymerase chain reaction (qPCR) was utilised to examine gene expression in wild type and transgenic placenta. Expression levels were compared between two wild type and two transgenic placentae from the same litter at each time point, and confirmed by repeating the analysis on samples from a second litter. Total RNA was isolated from the tissue to be analysed, complementary DNA (cDNA) generated by reverse transcription of mRNA, and used as the template for qPCR.

2.11.1 Isolation of RNA from tissue samples

Conceptuses were dissected as described in Section 2.4, with tissue from which RNA was to be extracted frozen on dry ice and stored at -80°C until genotypes were ascertained (Section 2.3). Whole tissue (up to ~100 mg) was homogenised in 1 ml RNA-Bee (Ams Biotechnology) with an RNase-Free pestle (VWR International) in a 1.5 ml microfuge tube. Chloroform (200 µl) was added to the homogenate, mixed vigorously and incubated on ice for five minutes. Tubes were centrifuged at 13,000 rpm for 15 minutes at 4°C, the aqueous phase removed to a fresh tube and 500 µl isopropanol added. The isopropanol precipitation was incubated at room temperature for between 5 and 10 minutes and tubes centrifuged at 13,000 rpm for 5 minutes at 4°C to pellet RNA. The RNA pellet was washed in 75% EtOH (1 ml), and subsequently allowed to air dry prior to being resuspended in 10 mM Tris (pH 8.0).

RNA-Bee is designed to isolate only RNA, and thus prevent DNA or protein contamination. As a precaution to avoid potential genomic DNA contamination, all RNA extracts were DNase-treated prior to use in generating cDNA. Approximately 30 µg of RNA was DNase-treated in a 1.5 ml microfuge tube as detailed in Table 2.14. RNA was incubated with RQ DNaseI (Promega) at 37°C for 45 minutes and RNA purified by phenol/chloroform extraction and NaOAc precipitation. Reaction volume was increased to 500 µl by addition of

RNase-free H_2O, and an equal volume (500 µl) of phenol added. Tubes were centrifuged at 13,000 rpm for 10 minutes at room temperature and the aqueous phase transferred to a fresh tube. An equal volume of chloroform was added and tubes centrifuged as before. The aqueous phase was removed to a separate tube and RNA precipitated in 10% of aqueous phase volume 3 M NaOAc (pH 5.2) and 2.5X aqueous phase volume 100% EtOH. Tubes were incubated at -20°C overnight and RNA pelleted by centrifugation at 13,000 rpm for 20 minutes at 4°C. The pellet was washed with 70% EtOH, allowed to air dry and resuspended in 25 µl of 10 mM Tris (pH 8.0). RNA quality was assessed by running 1 µl on a 2% TAE agarose gel, concentration determined using a NanoDrop as described previously, and adjusted to a concentration of 1 µg/µl in 10 mM Tris (pH 8.0).

	Volume (µl)
dH_2O	to 100
RQ DNase I 10X Buffer (Promega)	10
RNasin	1
RQ DNase I (Promega)	10
RNA	30 µg

Table 2.14: Reaction constituents for DNase treatment of RNA

Total RNA was DNase treated to remove potential DNA contamination prior to reverse transcription. The table shows constituent volumes for a single DNase treatment reaction.

2.11.2 Reverse transcription of cDNA from total RNA

Single stranded RNA cannot be used as template material for a PCR reaction. To enable relative transcript levels to be determined, cDNA was generated by reverse transcription of mRNA. First strand cDNA representing the entire mRNA transcriptome was synthesised from DNase treated total RNA, using random primers (also known as random hexamers) of 6 nucleotides length (Promega), and the Moloney Murine Leukemia Virus Reverse Transcriptase enzyme (M-MLV RT) (H-) (Promega). For each sample, two reactions were performed; one in the presence of the reverse transcriptase (RT+) and one without reverse transcriptase (RT-).

For each reaction, total RNA (5 μg) was incubated at 70°C for 10 minutes with 1 μl of random hexamers (0.5 μg/μl) (Promega) in a total reaction volume of 11 μl in RNase-Free water (Ambion). First strand cDNA transcription master mix (8 μl) (Table 2.15) was added to each reaction, and incubated at 37°C for 2 minutes. M-MLV (1 μl; 200 units) was then added to each RT+ reaction, transcription allowed to proceed for 1 hour at 37°C, with a final 15-minute incubation period at 70°C to denature the reverse transcriptase.

	Volume (μl)
dH_2O	2.5
5X First strand synthesis buffer	4
RNasin	0.5
10 mM dNTPs	1

Table 2.15: Reaction constituents for reverse transcription of cDNA

DNase treated total RNA was used as the template for the reverse transcription of cDNA. The table shows the constituent volumes for a single reverse transcription reaction.

RT+ and RT- reactions were diluted 1:19 in 10 mM Tris (pH 8). The purity of cDNA was assessed by PCR amplification of *β-Actin* or *Gapdh* (see Table 2.17 for primer sequences). Separate PCR reactions using RT+ and RT- reactions as template material were set up as outlined in Table 2.16. Thermocycler conditions were 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, then a 72°C final elongation step for 3 minutes. Half of the volume of each reaction was loaded on a 1.5% TAE agarose gel containing Ethidum Bromide (later SafeView) and visualised under UV. Successful reverse transcription resulted in the generation of the correct sized product from RT+ cDNA. The absence of genomic DNA contamination in the DNase-treated RNA was shown by the lack of product in RT- reactions; the presence of a band of any intensity indicated the presence of genomic DNA contamination. In such instances, RNA was incubated with DNase for an increased time, and the process of cDNA transcription repeated until RT- reactions were clean. Diluted cDNA was further diluted 1:4 in 10 mM Tris (pH 8) and stored at -20°C until required for qPCR.

	Volume (µl)
dH_2O	16.7
Buffer (10X) (Sigma)	2.5
$MgCl_2$ (25 mM) (Sigma)	2.0
dNTPs (10 mM each) (Roche)	0.8
Primers (Sigma Genosys)	1.0
Genomic Red Taq (Sigma)	1.0
Template cDNA (1:19 dilution)	1.0

Table 2.16: Reaction constituents for PCR analysis of cDNA

Quality and purity of cDNA was assessed from RT+ and RT- reactions in separate PCR reactions. The table shows constituent volumes for a single PCR reaction utilised to assess cDNA quality.

2.11.3 Design of primers for qPCR

Quantitative PCR is based on the same principle as standard PCR, except with product amplification directly and quantitatively measured after each cycle rather than a semi-quantitative analysis at the end of the entire procedure. The generation of PCR product was analysed by measuring the incorporation of the fluorescent dye "SYBR Green" (Finnzymes) into the PCR amplified product. Due to the increased sensitivity required for qPCR, primers were designed to generate a product of between 100- and 250 bp. When possible, forward and reverse primers were designed to anneal within adjacent exons, thus providing an additional level of control over potential genomic DNA contamination. Primers were designed to amplify from mRNA sequences available through NCBI using Primer3 (http://www.ncbi.nlm.nih.gov/sites/entrez). These mRNA sequences were first aligned to the associated genomic DNA sequence (also from NCBI) using the Spidey mRNA-to-genomic alignment program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html). The mRNA sequences were then annotated with exon-exon boundaries before being entered into Primer3 to design appropriate primers. Previously published primers were used for the reference genes *β-actin* (Ogawa *et al.*, 2003) and *Gapdh* (Wu *et al.* 2004). A summary of all qPCR primer sequences and their melting temperature is shown in Table 2.17, and their associated products in Table 2.18.

Target	ID	Sequence	T_m
Ascl2	R277	GAG CAG GAG CTG CTT GAC TT	59.9
	R278	CAG TCA GCA CTT GGC ATT TG	60.5
β-Actin	R150	CCT GTA TGC CTC TGG TCG TA	58.8
	R151	CCA TCT CCT GCT CGA AGT CT	59.6
Cdkn1c	R161	AGA GAA CTG CGC AGG AGA AC	60.0
	R162	TCT GGC CGT TAG CCT CTA AA	60.0
Kdr	R327	GGC GGT GGT GAC AGT ATC TT	60.0
	R328	GTC ACT GAC AGA GGC GAT GA	60.0
Gapdh	R148	CAC AGT CAA GGC CGA GAA TG	61.8
	R149	TCT CGT GGT TCA CAC CCA TC	62.0
Gcm1	R283	AGC CTG TGT TGA GCA GAC CT	60.1
	R284	TGT CGT CCG AGC TGT AGA TG	60.0
Hand1	R287	CGC CTG GCT ACC AGT TAC AT	60.2
	R288	GCG CCC TTT AAT CCT CTT CT	59.8
Igf2	R168	GTC GAT GTT GGT GCT TCT CA	59.8
	R169	AAG CAG CAC TCT TCC ACG AT	60.0
Phlda2	EG36	TCA GCG CTC TGA GTC TGA AA	60.0
	EG37	TCC TGG GCT CCT GTC TGA T	60.8
Slc2a3	R309	GAT CGG CTC TTT CCA GTT TG	59.8
	R310	CAA TCA TGC CAC CAA CAG AG	60.1
Slc3a2	R259	TGA TGA ATG CAC CCT TGT ACT TG	62.2
	R260	GCT CCC CAG TGA AAG TGG A	61.2
Slc22a18	EG84	TGA TGT CCA GTG TGC TCC AT	60.1
	EG85	AGA GTT CGG GTC AAT GGT TG	60.0
Slc38a2	R331	ACT CAT ACC CCA CCA AGC AG	60.0
	R332	CAC AAT CGC ATT GCT CAG AT	59.8
Slc38a4 (4-kb specific)	R323	TGA TTG GGA TGT TAG TCT GAG G	59.1
	R324	GGC CTG GGT TAA AAT GTG TG	60.2
Tpbpa	R285	TGA AGA GCT GAA CCA CTG GA	59.6
	R286	CTT GCA GTT CAG CAT CCA AC	59.5

Table 2.17: List of primers used for qPCR analysis of gene expression

The table summarises the sequences and melting temperature (T_m) of primer sets used in qPCR analysis of gene expression.

Target	mRNA accession number (NCBI)	Nucleotides amplified	Product	
			Size (bp)	T_m
Ascl2	NM 008554	1007 – 1182	176	84
β-Actin	NM 007393	502 – 761	260	85
Cdkn1c	NM 009876	1047 – 1187	141	86
Kdr	NM 010612	498 – 659	162	81
Gapdh	NM 008084	218 – 459	242	84
Gcm1	NM 008103	165 – 336	172	81
Hand1	NM 008213	770 – 967	198	85
Igf2	NM 010514	468 – 662	195	86
Phlda2	NM 009434	409 – 532	124	86
Slc2a3	NM 011401	183 – 358	176	81
Slc3a2	NM 008577	867 – 1049	183	81
Slc22a18	NM 008767	1140 – 1305	166	82
Slc38a2	NM 175121	1037 – 1163	126	79
Slc38a4	NM 027052	1829 – 1995	167	77
Tpbpa	NM 009411	25 – 174	150	82

Table 2.18: Summary of qPCR products

The table shows the amplicon size and melting temperature for qPCR products. Approximate product T_m was determined from melting curves from Opticon Monitor and is provided for reference only. Anti primer-dimer steps were performed at least 2 degrees below the melting temperature of the product.

2.11.4 Determination of relative gene expression

Gene expression was compared between wild type and transgenic tissue samples by qPCR. Relative transcript abundance was determined by measuring the incorporation of SYBR Green (DyNAmo HS SYBR Green qPCR kit) (Finnzymes) into the product detected by a Chromo4 Continuous Fluorescence Detector mounted on a PTC 200 Thermocycler (MJ Research). Thermocycler conditions were: 95°C for 15 min, followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec and an anti primer-dimer step performed at between 75°C to 82°C for 30 seconds. The anti-primer dimer step was performed to denature potential primer dimers, which may contribute to the measured fluorescence. The temperature of this step was set at least two degrees below the pre-determined melting temperature of the PCR product, to prevent denaturation of the specific product. Melting curve analysis was performed between 70°C and 95°C, at 0.5°C intervals. Fluorescence and melting curves were analysed using Opticon 3 software. Melting curves were examined to detect non-specific amplification or the presence of primer-dimers, evidenced by multiple peaks. A threshold fluorescence level was set in the early exponential phase for each assay. The point at which the fluorescence for each amplicon passed through this level was the cycle threshold (C_T), and was used to quantify relative transcript abundance.

Each assay consisted of a comparison between two wild type and two transgenic placentae from the same litter. Each sample was run in triplicate for each gene of interest (GOI) and reference genes *β-Actin* and *Gapdh.* The fold change of gene expression in transgenic placentae compared to wild type was calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). In each assay, the mean C_T for each sample was determined for all genes of interest and reference genes. The ΔC_T for each gene of interest was determined by subtracting the mean C_T for each reference gene from the mean C_T of each gene of interest. Separate ΔC_T values were calculated for each wild type and transgenic sample, and for each of the two reference genes, as below.

C_T WT GOI – C_T WT *β-Actin* and C_T WT GOI – C_T WT *Gapdh*

C_T Tg GOI – C_T Tg *β-Actin* and C_T Tg GOI – C_T Tg *Gapdh*

The $\Delta\Delta C_T$ value was then determined for each combination of wild type and transgenic sample comparisons, by subtracting the ΔC_T of the wild type from the ΔC_T of the transgenic sample. Fold change was determined by the $2^{-\Delta\Delta C_T}$ method relative to both reference genes as shown below:

$$2^{-[C_T \text{ Tg GOI} - C_T \text{ Tg } \beta\text{-Actin}] - [C_T \text{ WT GOI} - C_T \text{ WT } \beta\text{-Actin}]}$$

$$2^{-[C_T \text{ Tg GOI} - C_T \text{ Tg } Gapdh] - [C_T \text{ WT GOI} - C_T \text{ WT } Gapdh]}$$

For each assay, the mean fold change relative to the two reference genes was calculated, and the procedure repeated on the same samples to confirm the value. The same procedure was subsequently repeated on independent samples such that a total of four wild type and four transgenic samples were compared.

2.15 Investigating the post-natal phenotype

2.15.1 Weighing and dissection of adult mice

Adult mice were weighed weekly between P7 and 10 weeks of age, and subsequently every fifth week until one year of age. All mice weighed as part of this study were born within three weeks of each other, weaned at P28 and were co-housed with littermates of the same sex irrespective of genotype. No singly housed mice were included in this study. Mice were selected from this cohort at 10 weeks, 6 months and 1 year of age for fat pad and tibia measurements, thus accounting for the decreasing value of n over the course of the year. Mice were selected based on genotype, with at least four wild type and four transgenic mice dissected at each time point. Dissections were performed blind, with the identity and genotype of animals unknown until all animals had been dissected and weights collected.

Mice were killed by CO_2 inhalation at 10 weeks, 6 months and one year of age. Death was confirmed by the absence of response to pressure applied to the limbs. Blood was removed immediately following death by piercing the heart with a needle and removing blood into a syringe. Whole blood was immediately ejected into a heparinised tube, spun at 5,000 rpm for 5 minutes at room temperature and the serum removed and stored at -80°C until required.

Adipose tissue surrounding the intestines (mesenteric) and located behind the kidney (retroperitoneal) was dissected and weighed under advice from Dr Tim

Wells of Cardiff University. Adipose tissue was either fixed overnight in 4% PFA at 4°C or frozen immediately on dry ice and stored at -80°C. Kidneys, liver and pancreas were also dissected and weighed, with kidneys either fixed as above or frozen and stored at -80°C, and liver and pancreas PFA-fixed. RNA was extracted from frozen tissue as described in Section 2.11.1, cDNA generated as described in Section 2.11.2 and gene expression assessed by qPCR (Section 2.11.4). PFA-fixed tissue was washed in PBS and stored in 70% EtOH as described in Section 2.4, prior to embedding and sectioning by Derek Scarborough, and H&E staining as detailed in Section 2.6. The tibia from the rear left leg of each mouse was dissected under advice from Dr Tim Wells and the length measured.

2.15.2 Measurement of adipocyte cell diameter

Adipocyte size was estimated according to the method of Ashwell and colleagues (1976). Cell number was counted from three regions of H&E stained midline retroperitoneal adipose tissue sections viewed under a light microscope at 20X magnification. The "Touch Count" feature of analySIS software was utilised to count cell number by marking counted cells such that no cell was included twice or excluded from the analysis. In contrast to the published method, whereby cells were included if at least half of their area was contained in the region, in this case cells were included if they crossed the left or lower boundaries, but excluded if they crossed the right or upper boundary of the field of view. The effect of such an alteration is minimal, but excludes the need for a subjective decision as to which cells to include and which to exclude. For each animal the three cell counts were individually converted to a calculated cell diameter (see formula below), and the mean value for each animal determined. Overall wild type and transgenic mean estimated cell diameters were calculated from these values. Cell diameter was calculated according to the formula:

$$d' = \frac{2 \times \sqrt{A}}{m \times \sqrt{(n \times \pi)}}$$

Where d' is the calculated average cell diameter, A is the defined area of the region from which cells were counted (in μm^2) m is the final magnification of the section and n is the number of cells counted in the region. The value for A was calculated using the scale bar in the analySIS window, instead of from

photographs as described in the original method. Thus, as the defined area was equal to the actual area of the region, the final magnification was effectively 1X. Three regions were measured from three wild type and three transgenic animals at each age. An average calculated cell diameter was then determined for each individual, which was then used to calculate the mean cell diameter for wild type and transgenic animals at each stage.

2.16 Glucose tolerance testing

Glucose tolerance tests (GTT) were performed as described in Ma *et al.*, (2004). Mice were removed to a clean cage and fasted overnight (approximately 16 hours), with *ad libitum* access to water. Each mouse was weighed and a short (1-2 mm) piece of tail removed with a sterile sharp razor blade. The tail tip was gently bled by applying pressure from the base of the tail towards the tip, and blood collected in a HemoCue 201+ microcuvette (HemoCue) by capillary action. Excess blood was cleaned from the surface of the cuvette, which was placed in a HemoCue 201+ Glucose Analyser (HemoCue) to determine blood glucose concentration. Following determination of fasting baseline glucose levels, each mouse was injected with 2 mg/g filter-sterilised 2 M D-Glucose (Sigma) in dH_2O. Blood glucose concentration was measured every 30 minutes for two hours by gently removing coagulated blood from the tail tip and following the same procedure described above. A maximum of eight mice were subjected to the procedure on any single day. Following the procedure, animals were placed in a fresh cage, and closely monitored for at least 24 hours to ensure no adverse effects ensued. Throughout the procedure animals were handled with care to prevent undue distress being caused, which may adversely affect subsequent measurements. Glucose tolerance tests were performed on the same cohort of animals at 10 weeks, 6 months and 1 year of age.

2.17 Magnetic Resonance Imaging (MRI)

Mice were killed by CO_2-asphyxiation and scanned in a Bruker Biospec 94/20 (9.4T) scanner, equipped with a 72 mm internal diameter quadrature coil. Multislice spin echo scans with a TR of 3000 ms, TE of 12 ms and a flip angle of 180 degrees were performed. Sixty-four slices of 0.5 mm thickness were generated from a field of view of 8 cm by 4 cm, using a matrix size of 512 x 256,

giving a resolution of 156 μm/pixel. MRI scans were performed by Pawel Tokarczuk and Stephen Paisey at the EMRIC facility (Experimental MRI centre) within Cardiff School of Biosciences.

Two scans were performed on each mouse, one with fat suppression off and a second with fat suppression on. Fat suppressed scan images were subsequently subtracted from the non-suppressed scan images to generate a set of "fat-only" images from which total fat volumes were determined using Analyze MRI analysis software (Biomedical Imaging Resource, Mayo Clinic, Rochester MN). Whole animal volumes were determined from non-suppressed scan images, thus enabling a fat to body volume ratio to be determined for each mouse.

2.18 Enzyme-Linked ImmunoSorbent Assay (ELISA)

Serum concentration of insulin, leptin, and glucagon was determined by Enzyme-Linked ImmunoSorbent Assay (ELISA). Concentration of all three hormones was determined in the same reaction using 10 μl of serum (collected as described in Section 2.15.1) and the Mouse Endocrine Lincoplex Kit (Millipore). Each reaction was performed in duplicate on a 96-well microtitre plate alongside known standards. Absorbance was measured using a Luminex 100 plate reader, concentration of standards confirmed and used to determine the concentration of each hormone in each sample.

2.19 Statistical analysis of data

2.19.1 Choice of statistical test

Throughout the course of this investigation, data was collected from wild type and transgenic mice in order to compare a number of different variables, such as weight, area or volume. Appropriate statistical tests were chosen and applied to data sets to determine the probability that the two data sets (wild type and transgenic) were different. In each instance, two hypotheses were formulated; the null hypothesis (H_0) states that there is no difference between the two sets of data, whereas the alternative hypothesis (H_1) states that there is a difference between the two sets of data. The chosen statistical analysis tests whether or not the null hypothesis is true, by generating a probability value between 0 and 1.

The closer this value is to 0, the less likely the null hypothesis is to be true (Dytham 2003a).

Generally, one of two statistical tests was selected in order to compare the two data sets, dependent upon the distribution of the data. The Student's two-tailed *t*-test (henceforth the *t*-test) was used in instances where the spread of data followed a normal, or Gaussian distribution (Dytham 2003d). Alternatively, for data that did not follow a normal distribution, statistical analysis was performed using the Mann-Whitney *U* test (henceforth the Mann-Whitney test) (Dytham 2003d). The choice of statistical analysis was critical in order to arrive at the correct conclusion regarding the data.

To determine whether the *t*-test or Mann-Whitney test was used, data was first analysed using the one-sample Kolmogorov-Smirnov test (henceforth the Kolmogorov-Smirnov test), which tests whether the data is from a normal distribution with the same mean and variance (Dytham 2003d). As for the *t*-test and Mann-Whitney test, two hypotheses were formulated. H_0 states that the data follows a normal distribution with the same mean and variance as the sample data, whereas H_1 states that the data does not follow a normal distribution as described above. The Kolmogorov-Smirnov test tests whether or not the null hypothesis was true, by calculating a probability value as described above. As this test has low sensitivity when sample sizes are low (Dytham 2003c), it was used only when sample size was sufficiently large (in this instance, more than approximately 20 observations). When fewer than around 20 observations were made, the Kolmogorov-Smirnov test could not be applied, and thus the spread of data could not be tested. Neither could the data be assumed to follow a normal distribution. In such instances, data were analysed using the Mann-Whitney test, which makes no assumption as to the spread of data.

2.19.2 The *p*-value

All three statistical analyses calculate a probability, or *p* value, which relates to the probability of the null hypothesis being correct. The smaller this value is, the less likely H_0 is to be true, and thus the more likely we are to reject H_0 in favour of H_1. Generally, the minimum level at which H_0 was rejected (called the critical value) was 5%, such that H_0 was rejected when $p < 0.05$. The critical value is also referred to as the significance level of a test. Smaller *p* values are

associated with greater confidence in the conclusion for example, when $p < 0.01$, there is a 1% chance that H_0 is true. Thus, for the Kolmogorov-Smirnov test, if $p > 0.05$, the null hypothesis was not rejected, and the data was assumed to follow a normal distribution. Likewise, for both the *t*-test and Mann Whitney test, if $p < 0.05$, the null hypothesis was rejected, and the two data sets were said to be "significantly different".

The choice of statistical test was critical, as selection of the wrong type of analysis could result in the incorrect conclusion being drawn. Generally, two types of error are possible: a type I error results when the null hypothesis is incorrectly rejected, whereas a type II error occurs when the null hypothesis fails to be rejected (Dytham 2003b). A type I error results in the "false positive" identification of a statistically significant difference, where in fact there is no difference between data sets. At the 5% significance level, there is a 5% chance of making a type I error; using a lower significance level reduces the chance of a type I error, but increases the likelihood of a type II error. In general, the *p* value of each statistical test is given in the text and summarised on figures by the use of asterisks, the value to which they refer is defined in the figure legend.

2.19.3 The Chi-squared (χ^2) test

Genetic manipulation may directly or indirectly result in a varying degree of embryonic or post-natal lethality. The rules of Mendelian genetics dictate that by mating a transgenic male with a wild type female, transgenic and wild type progeny should be observed at equal frequencies at all stages. Further, as the transgene is not linked to a sex chromosome, transgenic and wild type animals should be present in a 1:1 ratio in both males and females. At all gestational stages examined, the observed number of wild type and transgenic animals was compared to the expected number using the Chi-squared test. The Chi-squared test tests the two hypotheses, H_0; that the observed number of animals is not significantly different to the expected number, and H_1; that there is a difference between the observed and expected numbers (Dytham 2003d). In all instances, the significance level was 5% and there was 1 degree of freedom, such that H_0 was not rejected if the calculated *p* value was < 3.841.

2.19.4 Normalisation of data

A number of environmental factors affect the growth of both embryo and placenta, which cannot be controlled for. Such factors result in a natural variation of weights of animals of the same genotype both within a litter (e.g. due to position in the uterus) and between litters (e.g. differences in litter size; slight variations in precise gestational age) (McLaren 1965). Initially raw data was analysed to identify any potential trends however, to account for the variation between individual weights, which may potentially mask or even introduce differences, all individual weights were "normalised". The individual weight of both wild type and transgenic embryos and placentae were adjusted by the ratio of wild type mean at that gestational age to the wild type mean of the appropriate litter.

$$\frac{\text{Mean of wild type}}{\text{Mean of wild types from litter}} \times \text{Individual value}$$

This method does not affect the overall wild type mean, but individual values were adjusted according to the above ratio thus reducing the standard deviation of both wild type and transgenic data sets. Data were presented in graphical form throughout this investigation, with graphs for both raw and normalised data, with error bars representing the standard deviation. As the standard deviation does not take into account the sample size, no conclusions can be drawn regarding statistical significance from whether or not standard deviation error bars overlap. Error bars representing standard deviation are used in favour of those representing standard error as they portray the actual variance of the data more accurately.

Chapter 3:

Characterising the placental phenotype of over-expressing *Phlda2* and *Slc22a18*

3.1 Overview

Over 100 imprinted genes have been identified in the mouse, of which a significant number are expressed in the placenta (Coan *et al.* 2005; Tycko 2006). The role of some of these genes in the placenta has been examined through knockout mouse models, the phenotypes of which are summarised in Table 3.1. Of the three genes located on the transgene used in this investigation, knockout models have been generated for *Cdkn1c* and *Phlda2*. *Cdkn1c* exhibits widespread, low level expression in the spongiotrophoblast and labyrinth. *Cdkn1c* null mice develop placentomegaly as a result of a proportional expansion of spongiotrophoblast and labyrinth layers, although there was no effect on giant cell or glycogen cell number (Takahashi *et al.* 2000). In contrast, *Phlda2* expression is restricted to the syncytiotrophoblast cells of the labyrinth. Loss of *Phlda2* causes placentomegaly as a consequence of an expanded spongiotrophoblast layer and increased glycogen cell abundance (Frank *et al.* 2002). *Slc22a18* mRNA is detected in E12.5 and E14.5 placenta (Dao *et al.* 1998), although the cell type(s) that express this gene have not yet been determined. The phenotype of *Slc22a18* null mice is yet to be reported.

Examination of the phenotype resulting from the loss of an imprinted gene is useful for assigning a function to a particular protein. Such knockout models however, provide little insight as to the advantage of acquiring an imprint at a specific gene or locus. Additionally, a number of human diseases result from over-expression of imprinted gene due to their loss of imprinting. Thus, over-expression animal models are vital in gaining a complete understanding of the role of imprinted genes in development of the embryo and placenta. Generation of such LOI models is significantly more difficult than creating a knockout model, with no approach able to reactivate the silent allele of a single imprinted gene. One approach involves the targeted deletion of the imprinting centre resulting in LOI of all genes in the domain. Consequently, there is loss of expression of some genes and two-fold expression of others making it difficult to assign a specific phenotype to a particular gene in the domain. Such mice have been generated for both the IC1 and IC2 sub-domains of mouse distal 7.

Gene	Null phenotype	Reference
Ascl2	Loss of spongiotrophoblast Disruption of labyrinth Expansion of giant cell layer	(Guillemot *et al.* 1994) (Tanaka *et al.* 1997)
Cdkn1c	Proportionate expansion of spongiotrophoblast and labyrinth	(Takahashi *et al.* 2000)
Cited1 (X-linked)	Irregular expansion of spongiotrophoblast Reduction and disruption of labyrinth	(Rodriguez *et al.* 2004)
Esx1 (X-lined)	Expansion of spongiotrophoblast Increased glycogen cell number Mislocalisation of spongiotrophoblast Disrupted vascularisation of labyrinth	(Li and Behringer 1998)
Grb10	Placentomegaly	(Charalambous *et al.* 2003)
Igf2	Disproportionate loss of labyrinth Disproportionate loss of glycogen cells from junctional zone	(Lopez *et al.* 1996) (Coan *et al.* 2008)
Igf2P0	Proportionate reduction of spongiotrophoblast and labyrinth	(Constância *et al.* 2002)
Igf2r	Placentomegaly	(Lau *et al.* 1994)
Mest/Peg1	Proportionate reduction of spongiotrophoblast and labyrinth	(Lefebvre *et al.* 1998)
Peg10	Complete loss of spongiotrophoblast Disrupted labyrinth development	(Ono *et al.* 2006)
Rtl1/Peg11	Impaired passive transport due to phagocytic attack of fetal capillaries	(Sekita *et al.* 2008)
Phlda2	Expansion of spongiotrophoblast Increased glycogen cell number	(Frank *et al.* 2002)

Table 3.1: Placental phenotype of imprinted gene knockout mice

The table shows the placental phenotype(s) associated with targeted disruption of specific imprinted genes. Maternally expressed genes are in red, paternally expressed genes are in blue, X-linked imprinted genes are in black. The paternally inherited X chromosome is preferentially inactivated in the extraembryonic tissue of female mice.

A mouse model of LOI at IC2 was generated by paternal inheritance of a disrupted *Kvdmr1*, resulting in expression of *Cdkn1c*, *Ascl2*, *Phlda2* and *Slc22a18* from both paternal and maternal alleles. Transgenic embryos exhibited symmetric growth restriction, which persisted into adulthood (Fitzpatrick *et al.* 2002). Furthermore, transgenic placentae were approximately 20% lighter than wild type (Salas *et al.* 2004). The *Kvdmr1* null mouse clearly demonstrates a role for *Kvdmr1* in suppressing paternal expression of a number of adjacent imprinted genes, and a role for these genes in placental and embryonic growth. However, the model is limited by an inability to accurately determine the relative contribution of each gene to the phenotype.

The contribution of *Phlda2* to the *Kvdmr1*$^{+/-}$ phenotype was examined by analysing the effect of restoring *Phlda2* gene dosage to normal. Mice that inherit a paternal *Kvdmr1* null allele in addition to a maternal *Phlda2* null allele possess a single active *Phlda2* allele, albeit on the paternal allele, whereas all other genes under the control of *Kvdmr1* remain biallelic. *Kvdmr1* null placentae were ~20% lighter than wild type. Restoration of *Phlda2* gene dosage to normal resulted in a ~10% reduction in placental weight compared to wild type and a ~50% rescue of embryonic growth. *Kvdmr1* null placentae also exhibit a reduced spongiotrophoblast region, which was restored to within the normal range by normalising *Phlda2* expression (Salas *et al.* 2004). Such comparative phenotypic analysis demonstrated a role for *Phlda2* in limiting expansion of the spongiotrophoblast, reciprocal to the *Phlda2* null mouse (Frank *et al.* 2002). However, the biallelic expression of other imprinted genes complicates a more detailed phenotypic analysis, particularly the investigation of adult phenotypes. Despite a partial rescue of embryonic growth restriction, restoring *Phlda2* gene dosage to normal did not rescue the symmetric growth restriction observed at P14 (Salas *et al.* 2004), which may to be due to over-expression of *Cdkn1c* (Andrews *et al.* 2007).

The aim of the work presented in this chapter was to further characterise the effect of over-expressing *Phlda2* and *Slc22a18* in the mouse placenta. Transgenic mice from line 10-15, which possess three copies of a modified BAC transgene from which *Phlda2* and *Slc22a18* are expressed were used to investigate the effect of over-expressing these genes in the placenta. Although

not necessarily an optimal model for LOI due to the presence of an additional three copies of the genes, such a transgenic model may allow the phenotypes associated with *Kvdmr1* null mice to be assigned to one or other of these genes. Furthermore, the excess expression from the transgene may amplify any associated phenotype. Gene expression was first examined using qPCR and *in situ* hybridisation with probes specific for *Phlda2* and *Slc22a18*. Placental weights were measured from E12.5 to E18.5 coupled with an examination of gross placental morphology from midline placental sections stained with haematoxylin and eosin staining. Labyrinth and spongiotrophoblast regions were directly measured from H&E stained sections from mid-late gestation placentae. Glycogen cell number was increased in *Phlda2* null placentae; glycogen content was assessed in transgenic placentae using periodic acid Schiff staining. Markers for several trophoblast cell types were analysed by *in situ* hybridisation, including those for giant cells, spongiotrophoblast and labyrinth.

3.2 Results

3.2.1 Appropriate spatial and temporal expression of *Phlda2* and *Slc22a18* in transgenic placentae

Southern blotting previously identified the presence of three copies of the modified BAC transgene in line 10-15 (John *et al.* 2001). Expression of both genes was reported to be at least 10-fold greater than endogenous levels in transgenic placentae at E14.5 and E16.5 on a mixed genetic background (Salas *et al.* 2004). Furthermore, in *Kvdmr1* null placenta, despite the presence of only two active copies of *Phlda2*, transcript levels were reported to be approximately four-fold the level of wild type placentae (Salas *et al.* 2004). Microarray analysis performed by Professor Tycko reported a 6.6 fold increase in *Phlda2* transcript levels in a comparison of three wild type and three transgenic placentae from line 10-15 on a mixed genetic background. Analysis of *Slc22a18* expression on this same microarray showed that expression was upregulated 5.4-fold in transgenic placentae. Previous analysis of expression of *Slc22a18* and in particular of *Phlda2* therefore suggested that transcript level was not correlated with the number of active gene copies present. Expression of these two genes in addition to *Cdkn1c* was further analysed by qPCR and *in situ* hybridisation.

Expression levels of *Phlda2*, *Slc22a18* and the inactivated *Cdkn1c* were first assessed in line 10-15 by qPCR at E12.5, when *Phlda2* levels are at their peak, and at E16.5, at which point only a few *Phlda2*-expressing cells remain in the labyrinth (Frank *et al.* 2002). The relative fold change for each gene in transgenic placentae compared to wild type at E12.5 and E16.5 is shown in Figure 3.1. Fold changes represent the mean fold change observed between a total of four wild type and four transgenic placentae, from two litters normalised to two reference genes, as described in Section 2.11. *Phlda2* was expressed at 3.70-fold (± 0.53) wild type levels at E12.5 and 3.33-fold (± 0.49) wild type levels at E16.5. Similarly, *Slc22a18* was expressed at 3.39-fold (± 0.87) the level of wild type at E12.5 and 2.73-fold (± 0.49) wild type at E16.5. Consistent with inactivation of *Cdkn1c* by insertion of the *β-galactosidase* reporter, *Cdkn1c* expression was comparable in wild type and transgenic placentae at E12.5 (1.18 ± 0.26) and E16.5 (1.03 ± 0.26).

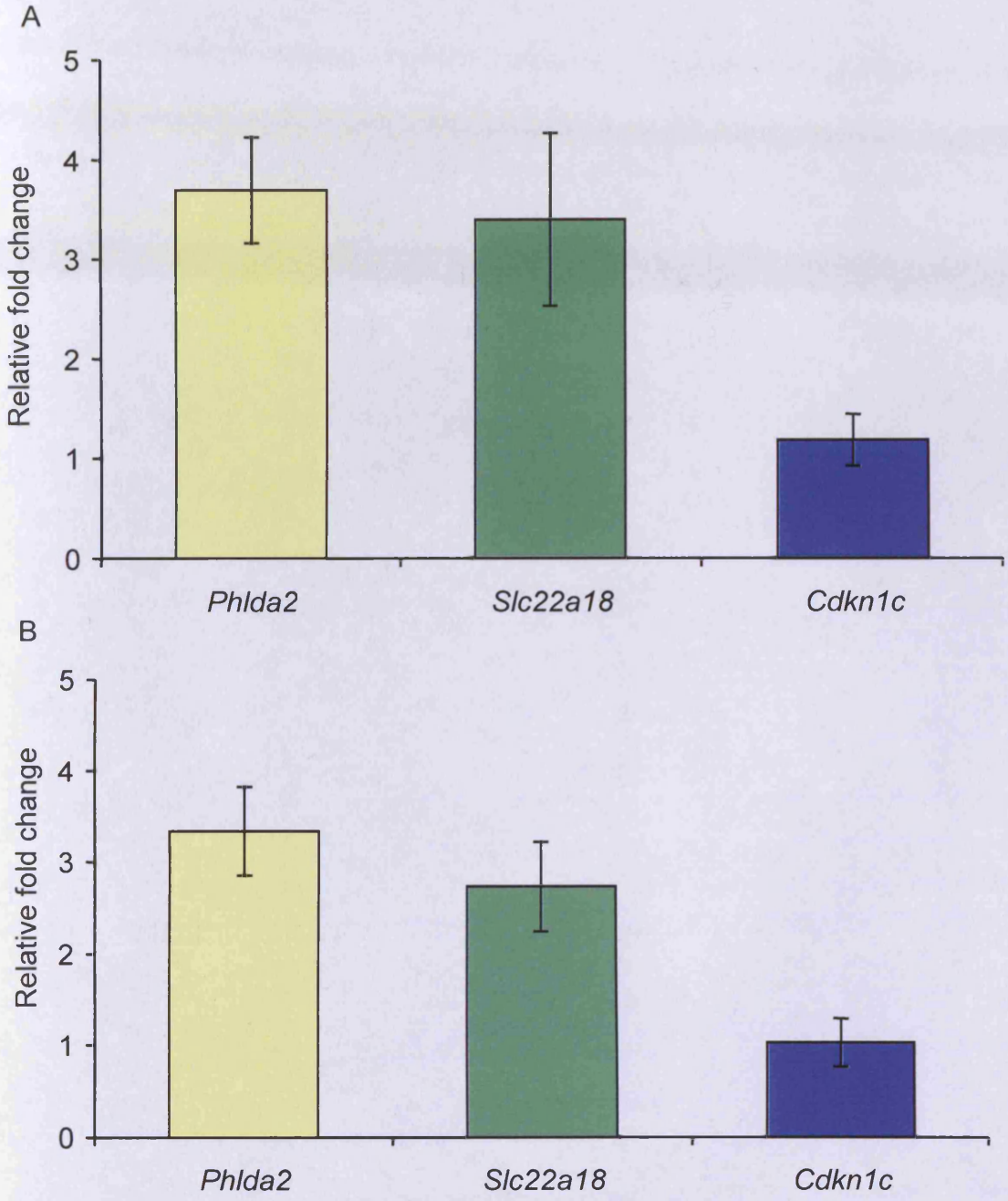


Figure 3.1: Relative expression levels of *Phlda2*, *Slc22a18* and *Cdkn1c*

Expression of *Phlda2*, *Slc22a18* and *Cdkn1c* was examined by qPCR to determine if expression levels correlated with transgene copy number. Relative expression levels were compared for the three genes located on the transgene at E12.5 and E16.5. Graph shows the relative fold change in gene expression determined by qPCR in transgenic placentae compared to wild type from line 10-15 on a pure 129/Sv background. Error bars represent standard deviation of fold change from four repeats.

The critical difference between the previously reported expression levels and the more recently generated data was the genetic background of the tissue. Expression of *Phlda2* was more than 10-fold endogenous levels on a mixed genetic background, with four-fold expression observed on a pure 129/Sv background. Intermediate levels of expression were observed in tissue collected from conceptuses between these two stages. Expression was next examined by *in situ* hybridisation. Visualisation of mRNA expression enabled the spatial expression pattern of both genes to be examined. Additionally, the *Cdkn1c* spatial expression pattern was assessed by *in situ* hybridisation. *Phlda2* and *Slc22a18* riboprobes were designed as described in Section 2.8.1, and hybridised to midline placental sections of appropriate gestational stages as detailed in Section 2.8.5 and 2.8.6.

Phlda2 is highly expressed during early gestation, with the number of *Phlda2*-expressing cells declining dramatically by E14.5, although a small number of labyrinth trophoblast cells continue to express the gene until late gestation (Frank *et al.* 2002; Frank *et al.* 1999; Qian *et al.* 1997). Expression of *Phlda2* was examined at E10.5 and E12.5 to compare relative expression levels during early gestation and to confirm that the number of cells expressing *Phlda2* is comparable in transgenic and wild type placentae. In contrast, little is known about the temporal or spatial expression of *Slc22a18*, and thus expression was initially assessed at E12.5.

Phlda2 expression in wild type and transgenic placentae was assessed by *in situ* hybridisation at E10.5 and E12.5. At both stages signal was only detected on slides hybridised with anti-sense probe, with no signal observed on slides hybridised with the sense probe and stain developed for the same duration. At E10.5, *Phlda2* expression was widespread in cells of the extraembryonic ectoderm of both wild type and transgenic placentae (Fig 3.2 A and B). By E12.5, expression became more restricted, with signal detected only in discrete cell clusters of the labyrinth of both wild type and transgenic placentae (Fig 3.2 C and D). There was no overt difference in the number of cells expressing *Phlda2* in transgenic placentae compared to wild type, although signal intensity was noticeably greater in transgenic placentae.

The localisation of *Slc22a18* expression in the mouse placenta has not previously been described, although placental expression has been reported at

E12.5 and E14.5, (Dao *et al.* 1998). As an organic cation transporter, expression would be expected in cells of the labyrinth. *In situ* hybridisation was utilised to observe the expression pattern of *Slc22a18* at E12.5 in both wild type and transgenic placentae, with widespread signal detected in the labyrinth and increased signal intensity in transgenic placentae compared to wild type (Fig 3.2 E and F). As predicted by qPCR analysis of *Cdkn1c* expression, no overt difference was observed in spatial expression between wild type and transgenic placentae at E14.5 (Fig 3.2 G and H).

To confirm that elevated expression of *Phlda2* resulted in increased translation and production of Phlda2 protein, immunohistochemical staining using an antibody specific for mouse Phlda2 was performed by Ben Tycko (Colombia University, New York). At both E10.5 and E12.5, Phlda2 protein signal was overtly increased in transgenic placentae and similarly localised in the anticipated regions (Fig 3.3).

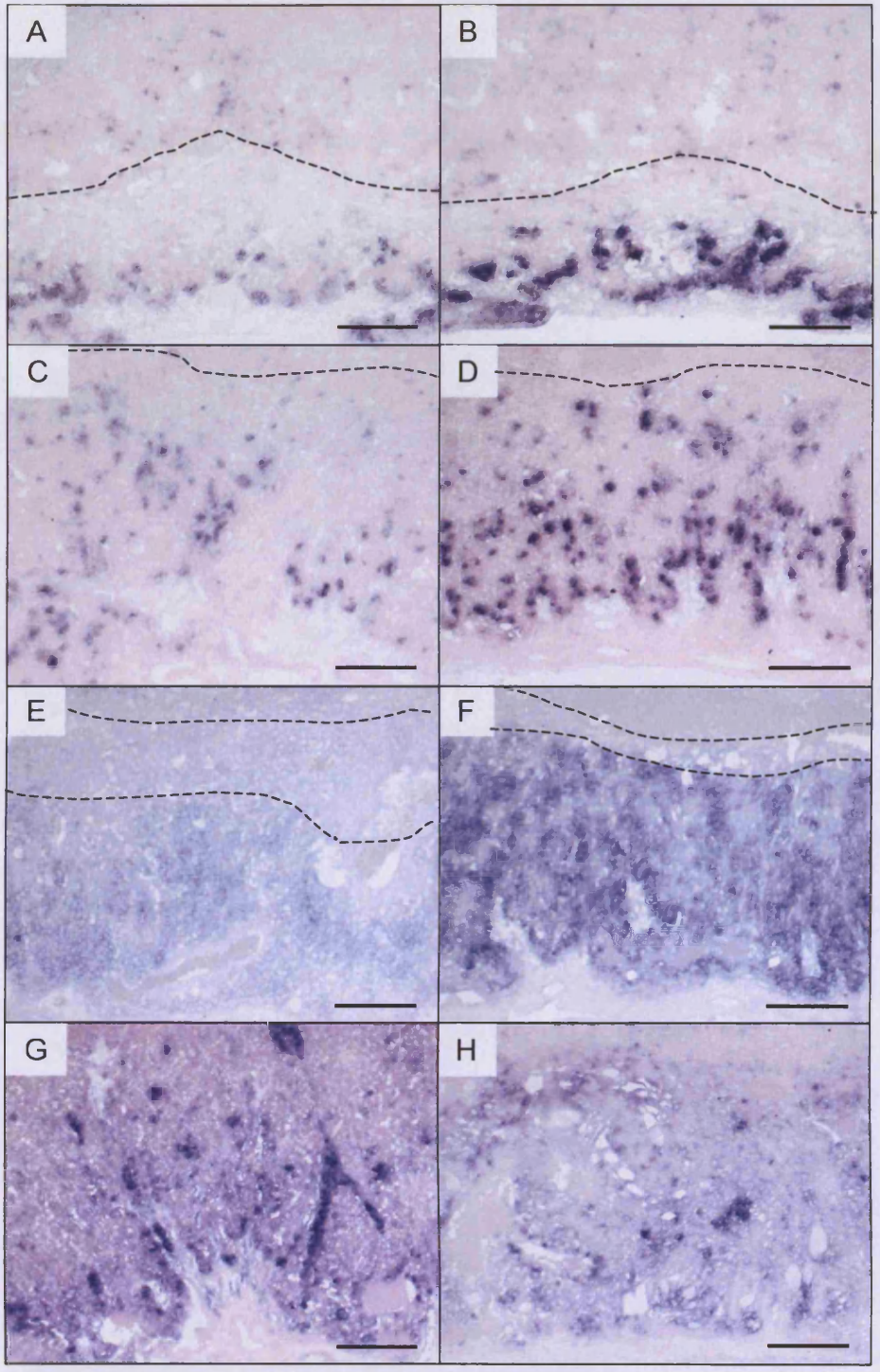


Figure 3.2: Expression of *Phlda2*, *Slc22a18* and *Cdkn1c* during early gestation

Spatial expression patterns for the three genes located on the transgene were assessed by *in situ* hybridisation. Midline placental sections from line 10-15 on a pure 129/Sv background were hybridised with an anti-sense probe specific for *Phlda2* (A-D), *Slc22a18* (E and F) and *Cdkn1c* (G and H). Sections from wild type placentae (left panels) and transgenic placenta (right panels) are at E10.5 (A and B), E12.5 (C-F) and E14.5 (G and H). Broken line indicates boundary between labyrinth (lower) and spongiotrophoblast (upper). Scale bar = 200 μm.

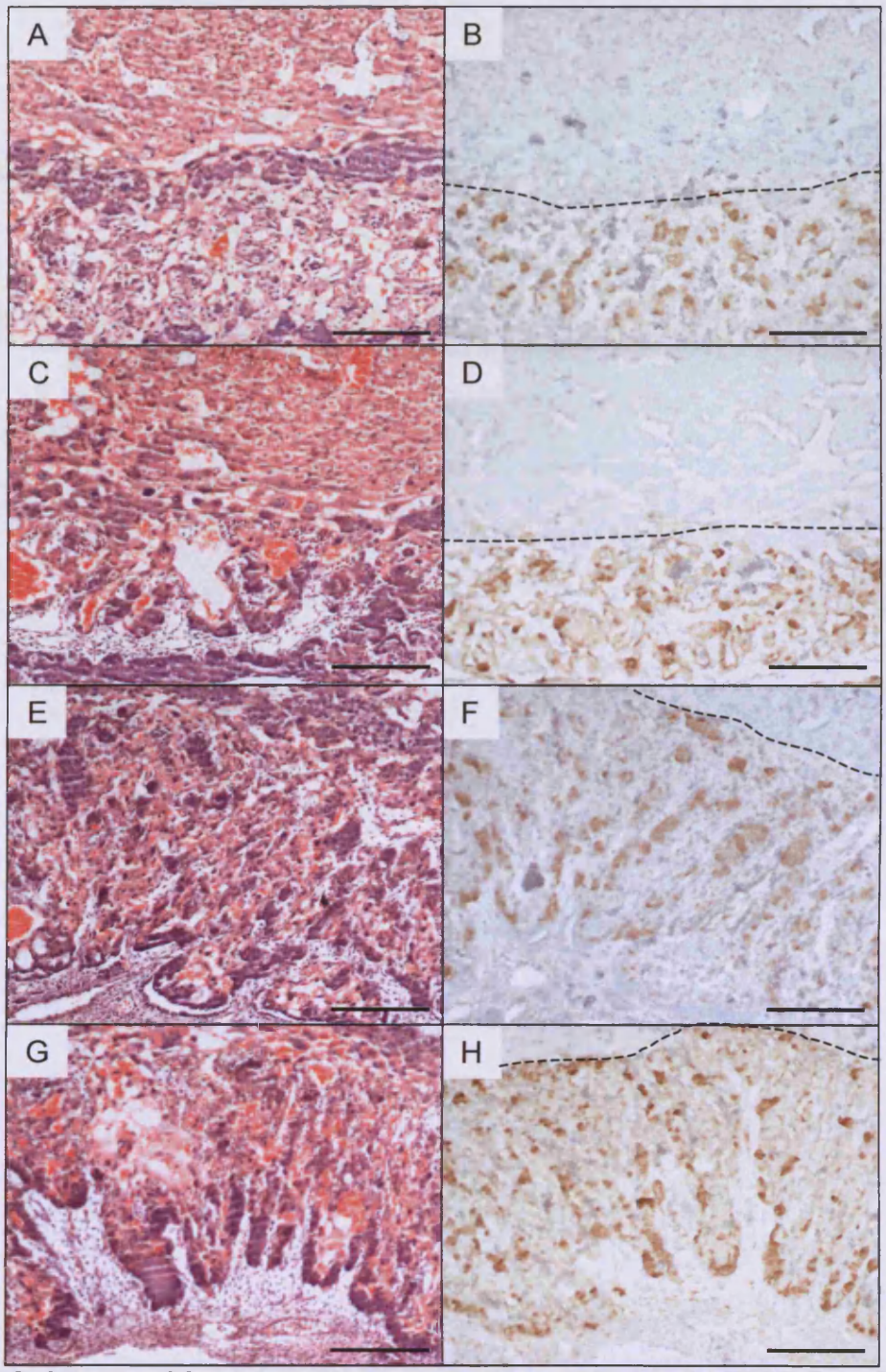


Figure 3.3: Immunohistochemical localisation of Phlda2 at E10.5 and E12.5

To confirm that elevated expression of *Phlda2* was associated with increased translation, immunohistochemical staining using an antibody specific to mouse Phlda2 was performed at E10.5 and E12.5. H&E stained midline placental sections (left panels) and Phlda2 IHC sections (right panels) from line 10-15 on a pure 129/Sv background. Figure shows wild type (A, B, E, F) and transgenic (C, D, G, H) at E10.5 (A-D) and E12.5 (E-H). Phlda2 protein signal was more intense in transgenic placentae, with no overt effect on number of positive cells. Broken line indicates boundary between labyrinth (lower) and spongiotrophoblast (upper). Scale bar = 200 μm.

3.2.2 Reduced weight of transgenic placentae

Targeted deletion of *Phlda2* in the mouse placenta causes placentomegaly, with a 22% increase in mean placental weight observed at E12.5 and a 27% increase at E16.5. Both $Phlda2^{neo}$ and $Phlda2^{LoxP}$ null lines displayed a comparable increase in placental size, which was further enhanced when crossed onto a pure C57BL/6 background (Frank *et al.* 2002). Initial characterisation of line 10-15 on a mixed genetic background revealed a reciprocal phenotype to the *Phlda2* KO, with a 25% reduction in placental weight at E14.5 and a 17% reduction at E16.5 (Salas *et al.* 2004).

Placental weights were gathered throughout gestation on a pure 129/Sv genetic background. Placental weight was examined from E12.5 until E18.5, in order to characterise the effect of *Phlda2* over-expression on early placental development and the extent of the placental phenotype prior to birth at E19. The earliest time-point at which accurate weight data could be obtained was E12.5, and thus placenta were weighed at E12.5, E13.5, E14.5, E16.5 and E18.5, generating a set of weight data representing stages between mid gestation and birth. At least six litters were collected at each stage, with data only utilised if at least two wild type and two transgenic embryos were in the same litter. Raw weight data was analysed and is presented in Figure 3.4A. Transgenic placentae were 15.4% lighter than wild type at E12.5 (51.1 mg ± 8.4 versus 42.3 mg ± 9.7; 9 litters; n = 74; $p = 7.8 \times 10^{-5}$), increasing to a 25.3% weight deficit at E13.5 (54.8 mg ± 9.0 versus 41.0 mg ± 9.6; 6 litters; n = 41; $p = 2.9 \times 10^{-5}$). At E14.5, transgenic placentae were 14.8% lighter (78.7 mg ± 12.5 versus 67.0 mg ± 11.5; 9 litters; n = 53; $p = 1.1 \times 10^{-3}$), and 17.6% lighter at E16.5 (82.3 mg ± 10.1 versus 67.8 mg ± 10.1; 8 litters; n = 58; $p = 1.05 \times 10^{-6}$). Prior to birth at E18.5, transgenic placentae were only 6.5% lighter than wild type, without achieving statistical significance (79.2 mg ± 11.4 versus 75.5 mg ± 18.3; 10 litters; n = 68; $p = 0.310$).

Individual raw weights of wild type placentae exhibited a standard deviation of up to 17% the mean weight for each gestational stage, with greater variation observed in transgenic placentae. Such variations in weight may be caused by a number of factors that could not be controlled for, such as litter size, exact gestational age, position of the conceptus in the uterine horn, maternal

health and other environmental factors (McLaren 1965). To prevent such factors from potentially disguising or influencing apparent weight phenotypes, individual weights of both wild type and transgenic embryos were adjusted by the ratio of the mean wild type weight for the gestational stage to the mean wild type weight for the litter, as described in Section 2.19.4. Such manipulation did not alter the mean wild type weight, but reduces the standard deviation of both wild type and transgenic data sets. Mean normalised embryonic weights of wild type and transgenic embryos are presented in Figure 3.4B, with error bars representing standard deviation of normalised data. The mean transgenic weight is presented as a percentage of mean wild type weight for each gestational stage.

Following normalisation of placental weight data, transgenic placenta were 15.4% lighter than wild type at E12.5 (51.1 mg ± 6.5 versus 43.2 mg ± 9.7; 9 litters; n = 74; $p = 9.1 \times 10^{-5}$), increasing to a 26.2% difference at E13.5 (54.8 mg ± 6.2 versus 40.5 mg ± 8.6; 6 litters; n = 41; $p = 5.5 \times 10^{-7}$). Transgenic placentae were 14.9% lighter at E14.5 (78.7 mg ± 6.5 versus 66.9 mg ± 6.4; 9 litters; n = 53; $p = 3.1 \times 10^{-8}$), and 16.6% lighter at E16.5 (82.3 mg ± 6.3 versus 68.7 mg ± 11.3; 8 litters; n = 58; $p = 3.99 \times 10^{-7}$). At E18.5, transgenic placentae were observed to be only 9% lighter than wild type (79.2 mg ± 7.2 versus 72.5 mg ± 11.6; 10 litters; n = 68; $p = 0.0018$).

Wild type placentae reached their maximum weight at E14.5, exhibiting an increase of approximately 50% between E12.5 and E14.5, with the majority of this weight gain occurring from E13.5. Wild type placental weight did not significantly alter between E14.5 and E18.5. Transgenic placentae exhibited a similar growth pattern, with a rapid gain in weight between E13.5 and E14.5. In contrast to wild type placental growth, the weight of transgenic placentae increased by approximately 10% between E16.5 and E18.5, such that the extent of the weight difference at E18.5 was considerably less than at E14.5. Furthermore, the weight of individual transgenic placenta was considerably more variable than that of wild type, particularly at later gestational stages.

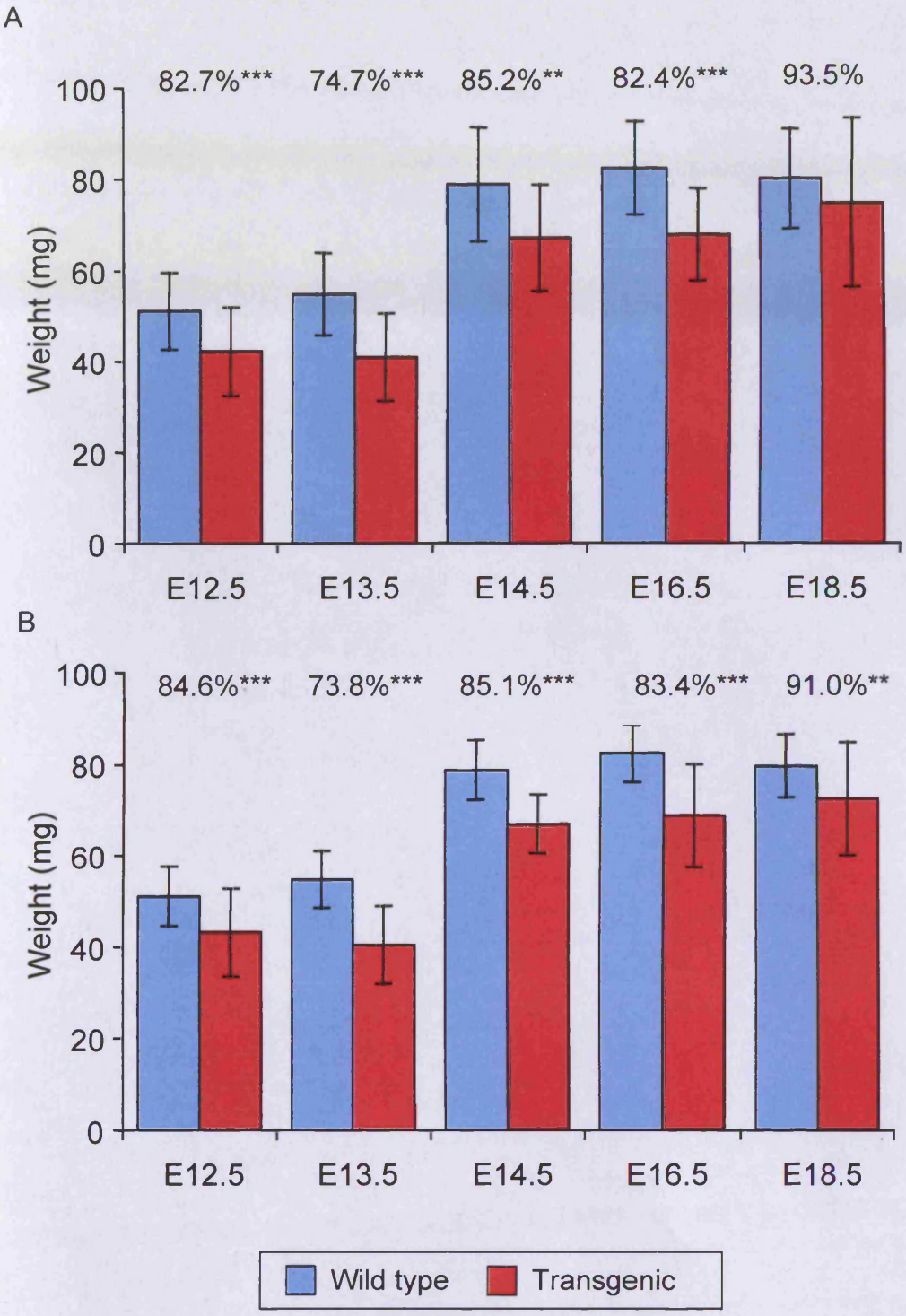


Figure 3.4: Placental weights from line 10-15 between E12.5 and E18.5

Placental weight was examined throughout gestation to examine the effect of over-expressing *Phlda2* and *Slc22a18* in the placenta. Graphs show A) raw and B) normalised mean weight of wild type and transgenic placenta at the indicated stages during gestation from line 10-15 on a pure 129/Sv background. Error bars show standard deviation. Significance level according to the Student's *t*-test indicated by asterisks: ** $p < 0.01$; *** $p < 0.001$.

The number of wild type and transgenic conceptuses observed at each developmental stage was compared to the number that would be expected according to Mendelian ratios using the Chi-squared (χ^2) test. *P* values for each analysis are shown in Table 3.2. At all stages examined the probability value was less than the critical value of 3.841, thus indicating that there was no difference between the expected number and observed frequency. The observed n values include litters in which there were fewer than 2 individuals of each genotype, and thus were excluded from weight analysis.

Stage	Wild type		Transgenic		$P(\chi^2)$
	Observed n	Expected n	Observed n	Expected n	
E12.5	43	44	45	44	0.046
E13.5	28	34	40	34	2.120
E14.5	39	41	43	41	0.195
E16.5	43	39	35	39	0.821
E18.5	48	44.5	41	44.5	0.551

Table 3.2: *P*-values for Chi-squared test

A factor to be considered when investigating transgenic mouse models is an unexpected effect on embryonic viability, resulting in a reduced number of transgenic progeny compared to the expected frequency. The Chi-squared test was performed to determine if the observed frequency of wild type and transgenic progeny at each stage during gestation differed significantly from the expected frequency predicted by Mendelian inheritance. At the five stages examined, there was no deviation from the expected frequency of transgenic progeny.

3.2.3 Reduced spongiotrophoblast in transgenic placentae

In *Phlda2* null placentae, the ratio of spongiotrophoblast to labyrinth cross-sectional area was increased by approximately 50% at E16.5, as a consequence of the disproportionate expansion of the spongiotrophoblast, with a slight increase in labyrinth area (Frank *et al.* 2002). Over-expression of *Phlda2* has indirectly been shown to restrict development of the spongiotrophoblast. Mice that inherit a paternal deletion of *Kvdmr1*, and thus over-express *Phlda2* in addition to *Cdkn1c*, *Ascl2* and others (Fitzpatrick *et al.* 2002), exhibit a 24% reduction in the spongiotrophoblast to labyrinth ratio at E14.5. When *Phlda2* expression levels were restored to normal by maternal inheritance of a *Phlda2*

null allele in addition to the paternally inherited *Kvdmr1* null, the spongiotrophoblast to labyrinth ratio was almost entirely rescued, with a 6% reduction compared to wild type placentae observed at E14.5 (Salas *et al.* 2004). Thus, over-expression of *Phlda2* accounts for around 75% of the alteration to the spongiotrophoblast to labyrinth ratio at this stage. A similar pattern of results was reported at E16.5, however the margins were greatly reduced and thus statistical significance was not achieved between the comparisons as above.

The gross morphology of placentae between E10.5 and E18.5 was examined from H&E stained midline sagittal sections (Fig 3.5). Transgenic placentae were visibly smaller from as early as E10.5, although accurate weight data could not be obtained at this early stage. At this stage, the ectoplacental cone and extraembryonic ectoderm regions could not be distinguished morphologically at low magnification. At E12.5, the spongiotrophoblast and labyrinth regions of the placentae could be distinguished under low magnification on a light microscope. At E14.5, H&E staining enabled the spongiotrophoblast and labyrinth regions to be readily distinguished under low magnification on a dissection scope. The spongiotrophoblast was generally more darkly stained and exhibited large maternal blood lacunae in wild type placentae, which were markedly reduced or absent in transgenic placentae. Wild type placentae at E16.5 were essentially the same as E14.5, although with a larger labyrinth layer at the expense of a smaller spongiotrophoblast with reduced maternal blood spaces. Similarly, transgenic placentae exhibited a reduced spongiotrophoblast with smaller maternal blood spaces. At E18.5, the size of the spongiotrophoblast was reduced in wild type placentae, and was generally more compact than at earlier stages. In transgenic placentae, the spongiotrophoblast region was barely distinguishable at E18.5.

Spongiotrophoblast and labyrinth regions were previously quantified at E14.5 and E16.5 from *Kvdmr1* null and *Kvdmr1/Phlda2* null placentae. Similarly, these regions were quantified at these stages in wild type and transgenic placentae from line 10-15. At E14.5, the volume of both regions was determined and compared to areas measured from midline sections to assess whether midline area measurements provided an adequate approximation of volume. Midline areas were also measured at E16.5.

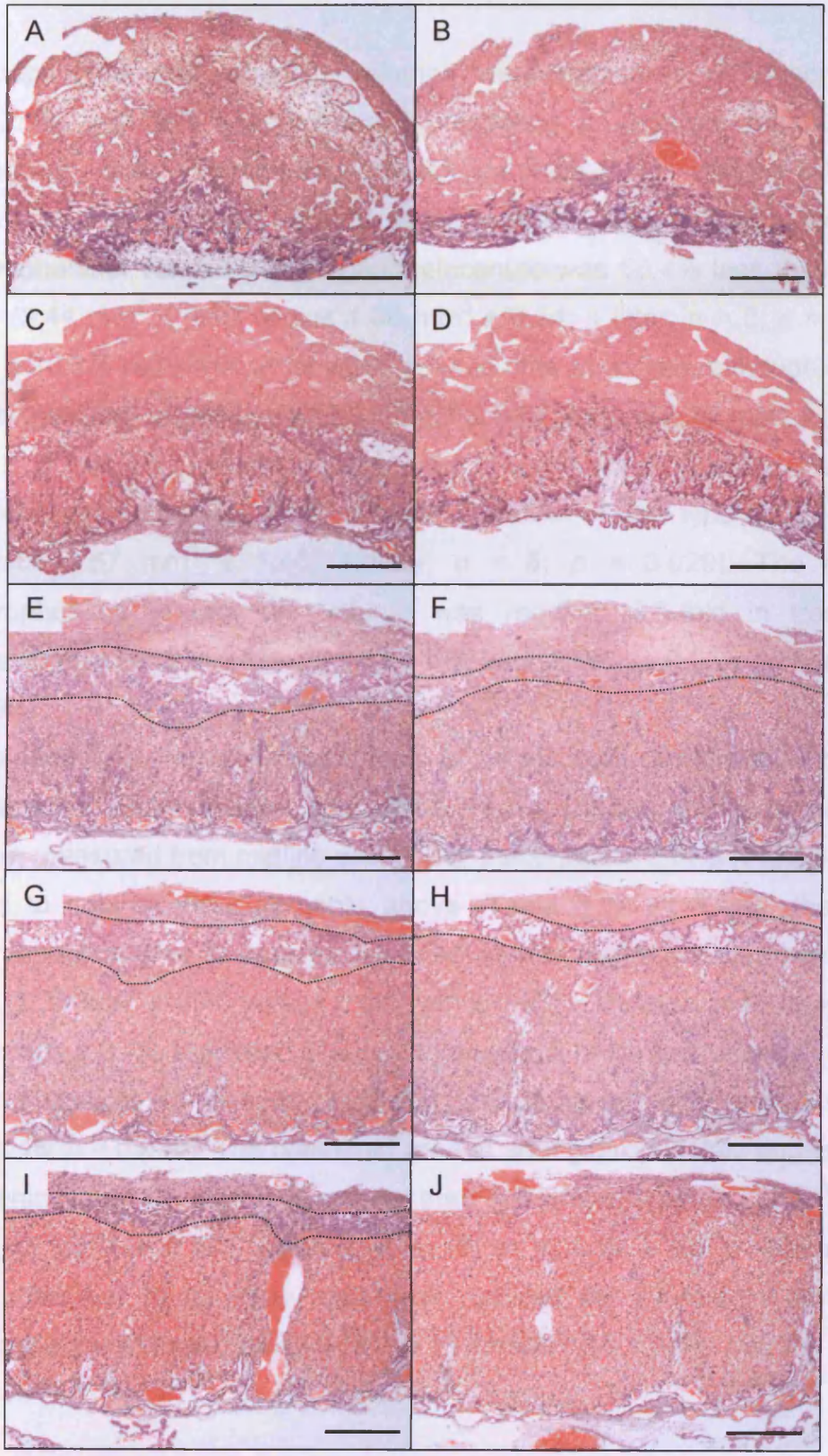


Figure 3.5: H&E staining of midline placental sections

Gross placental morphology was assessed from H&E stained midline sections of wild type (left panels) and transgenic (right panels) at (from top) E10.5, E12.5, E14.5, E16.5 and E18.5. Placentae were from line 10-15 on a pure 129/Sv background. Dashed lines denote boundary of spongiotrophoblast, which was overtly reduced in transgenic placentae. Scale bar = 500 μm.

Spongiotrophoblast and labyrinth volumes were measured as described in Section 2.10, with the total volume representing a separate measurement encompassing both spongiotrophoblast and labyrinth regions. Volume measurements for each region are displayed in Figure 3.6A. At E14.5, the mean spongiotrophoblast volume of transgenic placentae was 60.4% less than that of wild type (3.44 mm^3 ± 0.60 versus 1.36 mm^3 ± 0.34; 1 litter; n = 8; p = 0.029), whereas a 10.3% reduction in labyrinth volume was observed, although this did not reach statistical significance (9.21 mm^3 ± 0.77 versus 8.26 mm^3 ± 1.13; 1 litter; n = 8; p = 0.200). The combined volume of spongiotrophoblast and labyrinth of transgenic placentae was 24.5% smaller than that of wild type (12.81 mm^3 ± 1.20 versus 9.67 mm^3 ± 1.40; 1 litter; n = 8; p = 0.029). The ratio of spongiotrophoblast to labyrinth volume was reduced 2.3-fold in transgenic placentae compared to wild type (0.37 ± 0.056 versus 0.16 ± 0.025; 1 litter; n = 8; p = 0.029).

To determine if area measurements at the placenta midline could be used as an estimate of region volume, spongiotrophoblast and labyrinth cross sectional areas were measured from midline sections of placentae at E14.5 (Fig 3.6B), and compared to volume measurements above (Table 3.3). At E14.5, the mean spongiotrophoblast area of transgenic placentae was 59.9% smaller than that of wild type (1.35 mm^2 ± 0.40 versus 0.54 mm^2 ± 0.13; 3 litters; n = 18; p = 6.28 x 10^{-5}), whereas a 2.7% reduction in labyrinth area was observed, although this did not reach statistical significance (3.66 mm^2 ± 0.36 versus 3.56 mm^2 ± 0.59; 3 litters; n = 18; p = 0.930). The combined area of spongiotrophoblast and labyrinth of transgenic placentae was 19% smaller than that of wild type (5.10 mm^2 ± 0.41 versus 4.13 mm^2 ± 0.58; 3 litters; n = 18; p = 1.19 x 10^{-3}). The ratio of spongiotrophoblast to labyrinth area was reduced by 2.4-fold in transgenic placentae compared to wild type (0.38 ± 0.13 versus 0.16 ± 0.05; 3 litters; n = 18; p = 4.4 x 10^{-4}).

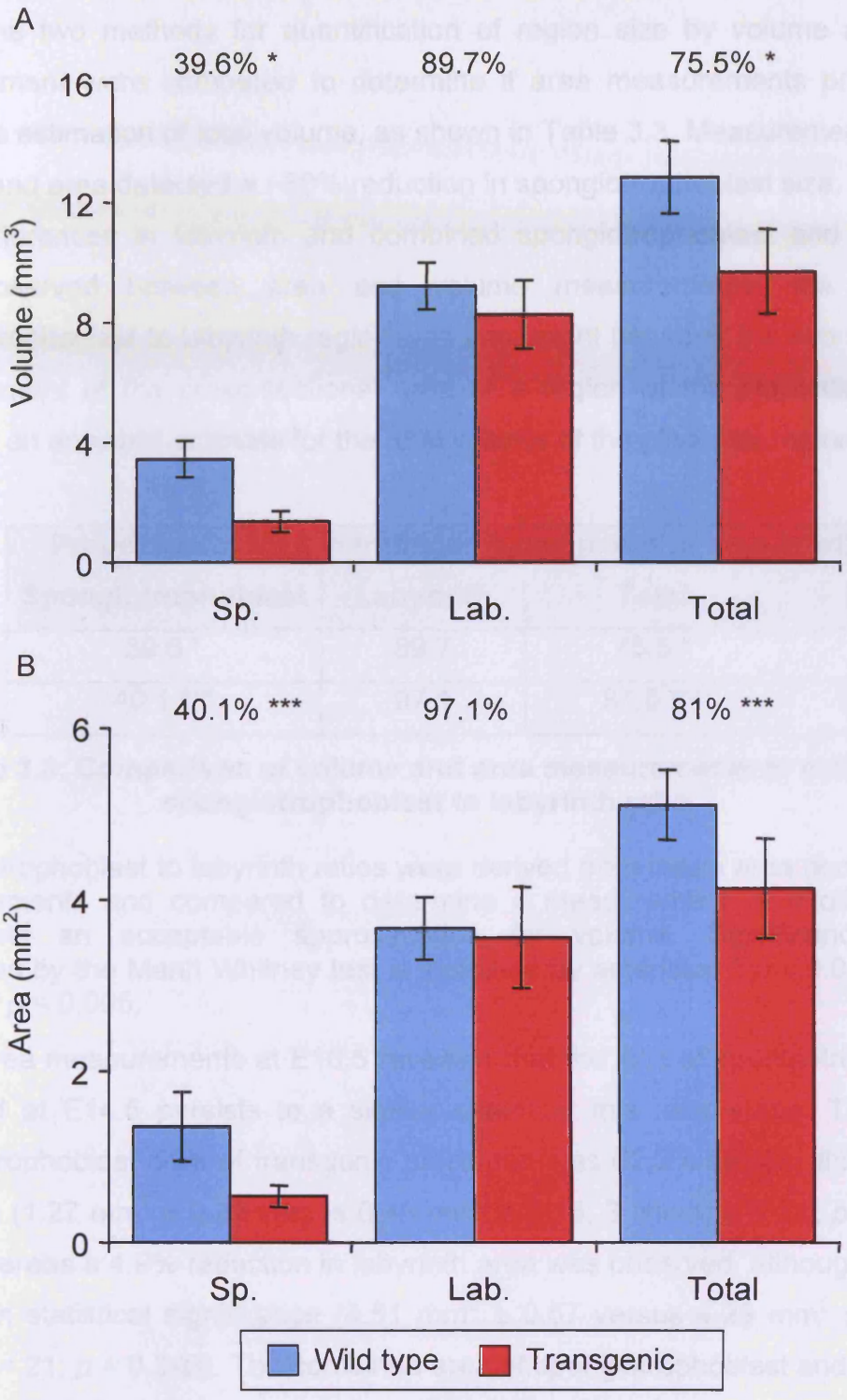


Figure 3.6: Measurement of spongiotrophoblast and labyrinth at E14.5

Spongiotrophoblast and labyrinth size was quantified at E14.5 by measurement of entire volume and midline areas. Graphs show A) raw volume and B) raw area of spongiotrophoblast (Sp.), labyrinth (Lab.) and combined spongiotrophoblast and labyrinth regions (Total) for wild type and transgenic placentae of line 10-15 on a pure 129/Sv background at E14.5. Error bars show standard deviation. Significance level calculated by the Mann Whitney test is indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

The two methods for quantification of region size by volume and area measurement were compared to determine if area measurements provide an adequate estimation of total volume, as shown in Table 3.3. Measurement of both volume and area detected a ~60% reduction in spongiotrophoblast size. Although slight differences in labyrinth and combined spongiotrophoblast and labyrinth were observed between area and volume measurements, the ratio of spongiotrophoblast to labyrinth region was consistent between the two methods. Measurement of the cross-sectional area of a region at the placental midline provides an accurate estimate for the total volume of the placental region.

	Proportion of area in transgenic compared to area in wild type			
	Spongiotrophoblast	**Labyrinth**	**Total**	**Ratio**
Volume	39.6 *	89.7	75.5 *	2.3 *
Area	40.1 ***	97.1	81.0 ***	2.4 ***

Table 3.3: Comparison of volume and area measurements to estimate spongiotrophoblast to labyrinth ratio

Spongiotrophoblast to labyrinth ratios were derived from mean area and volume measurements and compared to determine if measurement of midline area represents an acceptable approximation for volume. Significance level calculated by the Mann Whitney test is indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Area measurements at E16.5 revealed that the loss of spongiotrophoblast observed at E14.5 persists to a similar extent at this later stage. The mean spongiotrophoblast area of transgenic placentae was 62.3% smaller than that of wild type (1.27 mm^2 ± 0.32 versus 0.48 mm^2 ± 0.15; 3 litters; n = 21; $p = 5.67 \times 10^{-6}$), whereas a 4.9% reduction in labyrinth area was observed, although this did not reach statistical significance (4.51 mm^2 ± 0.57 versus 4.29 mm^2 ± 0.87; 3 litters; n = 21; $p = 0.349$). The combined area of spongiotrophoblast and labyrinth of transgenic placentae was 19% smaller than that of wild type (5.67 mm^2 ± 0.91 versus 4.77 mm^2 ± 0.87; 3 litters; n = 21; $p = 0.043$). The ratio of spongiotrophoblast to labyrinth volume was reduced 2.4-fold in transgenic placentae compared to wild type (0.28 ± 0.06 versus 0.12 ± 0.04; 3 litters; n = 21; $p = 5.67 \times 10^{-6}$).

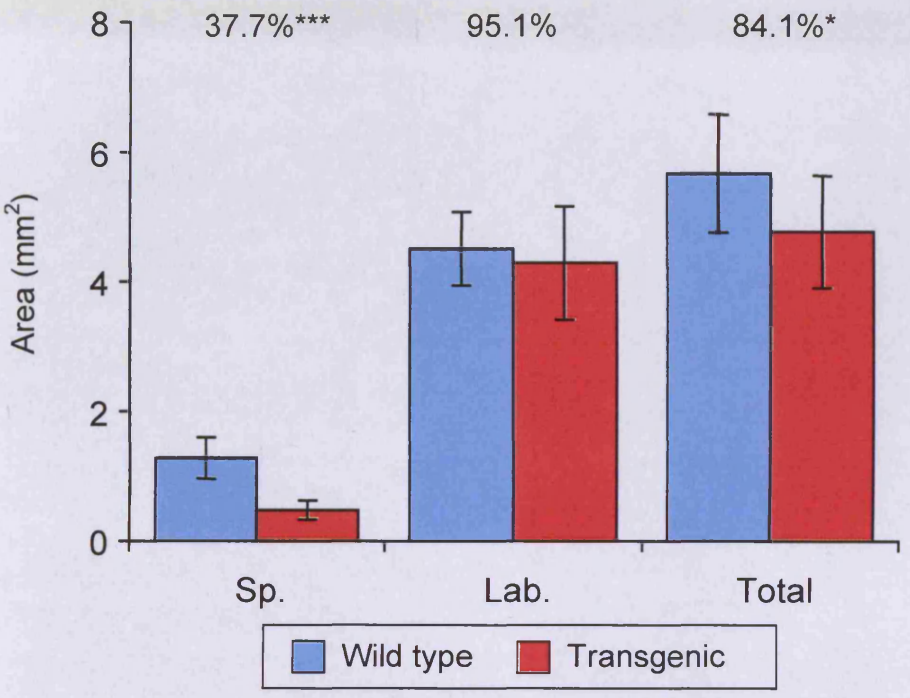


Figure 3.7: Measurement of spongiotrophoblast and labyrinth areas

Spongiotrophoblast and labyrinth areas were further quantified at E16.5 to confirm the persistence of the phenotype at this stage. Graph shows mean raw areas of spongiotrophoblast (Sp.), labyrinth (Lab.) and combined spongiotrophoblast and labyrinth regions (Total) of wild type and transgenic placentae from line 10-15 on a pure 129/Sv background at E16.5. Error bars show standard deviation. Significance level calculated by the Mann Whitney test is indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Phlda2 is predominantly expressed during early gestation and thus it is likely that the loss of spongiotrophoblast observed at E14.5 and E16.5 was a consequence of perturbed placental development at earlier gestational stages. It was not possible to clearly distinguish spongiotrophoblast and labyrinth on H&E stained sections prior to E14.5. Expression of the *trophoblast specific protein alpha* gene (*Tpbpa*) gene is restricted to cells of the junctional zone in rodent placenta and can thus be used to clearly distinguish this region in wild type and transgenic placenta (Lescisin *et al.* 1988). The *Tpbpa* gene shares homology to cathepsin propeptide genes, which encode inhibitors of cathepsin proteases, suggesting a role for *Tpbpa* in also inhibiting these proteases that function in various aspects of placentation including proliferation, differentiation and vascularisation (Rawn and Cross 2008; Screen *et al.* 2008)

The expression pattern of *Tpbpa* was examined by *in situ* hybridisation of midline placental sections at E10.5, E12.5, E14.5, E16.5 and E18.5 in order to assess the relative size of the junctional zone at these stages. The extent of *Tpbpa* staining was markedly reduced in transgenic placentae at E10.5 (Fig 3.8 A and B), although signal was correctly localised to cells of the ectoplacental cone. A similar reduction in signal was observed at E12.5, with the spongiotrophoblast appearing as a more compact region in transgenic placentae (Fig 3.8 C and D). Analysis of *Tpbpa* expression by qPCR at E12.5 revealed a statistically significant 2.1-fold reduction in transcript level in transgenic placentae (± 0.04; n = 8; $p = 0.014$). The extent of *Tpbpa* staining was similarly reduced at E14.5 and E16.5 (Fig 3.8 E - H). At E18.5, the spongiotrophoblast appeared as a distinct zone in wild type placentae, albeit reduced in size compared to E16.5, with a clear boundary existing between this and the labyrinth region (Fig 3.8 I). In transgenic placentae however, this boundary between the two zones was completely absent, with only a few *Tpbpa* positive cells localised in the anticipated area, the majority of *Tpbpa* positive cells were instead dispersed throughout the labyrinth region of transgenic placentae (Fig 3.8 J). *Tpbpa* positive cells were observed to mislocalise in the labyrinth layer of three transgenic placentae at E18.5, with no such mislocalisation seen in three wild type placentae at the same stage. Similarly, a clear spongiotrophoblast boundary was not observed at E18.5 in an additional five transgenic placenta stained with H&E.

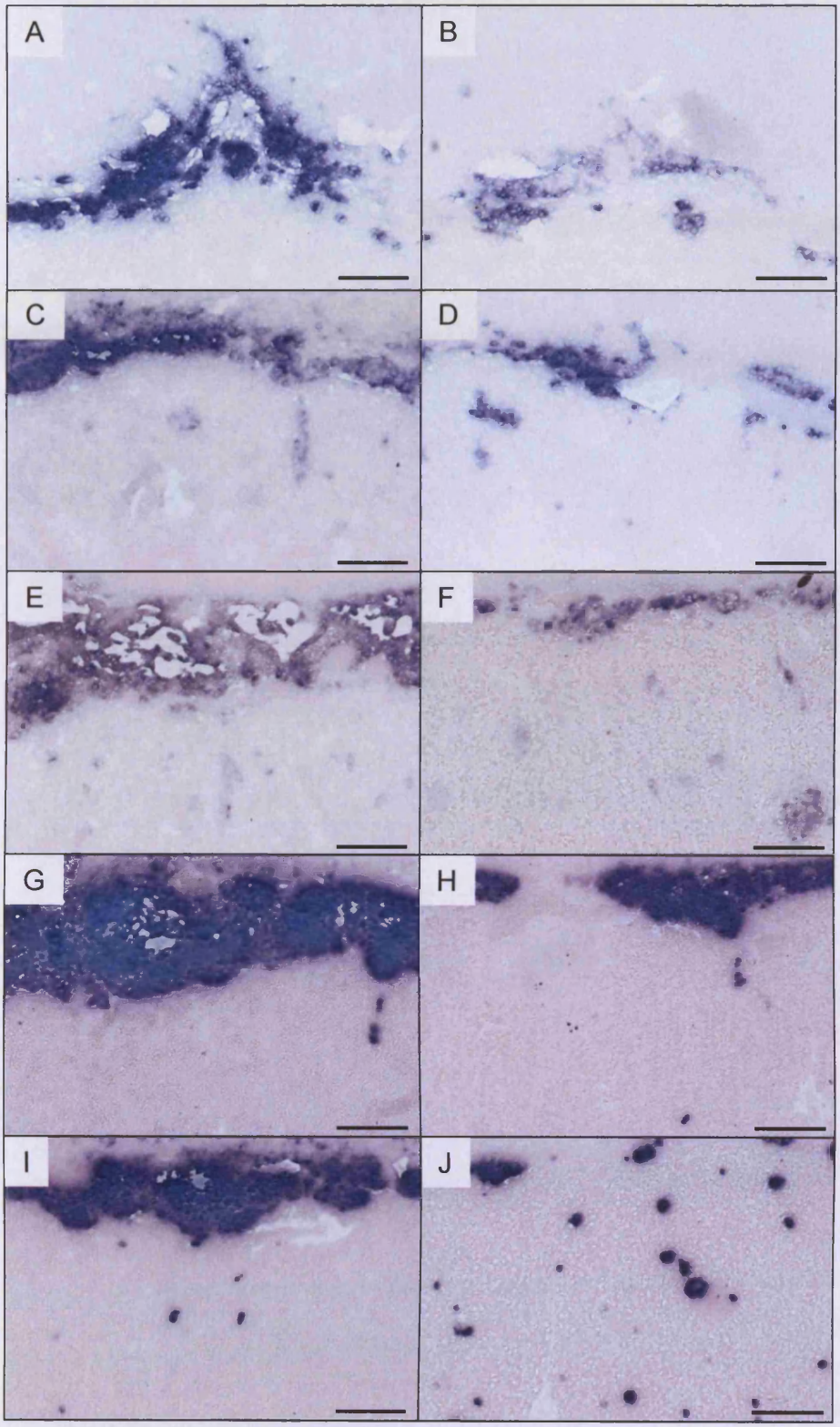


Figure 3.8: Spatial and temporal expression pattern of *Tpbpa*

A direct examination of junctional zone cells was performed throughout gestation by hybridisation of placental sections with the spongiotrophoblast-specific riboprobe *Tpbpa*. Figure shows midline placental sections from line 10-15 on a pure 129/Sv background. Wild type (left panels) and transgenic (right panels) at (from top) E10.5, E12.5, E14.5, E16.5 and E18.5 hybridised with the *Tpbpa* riboprobe. Scale bar = 200 μm.

3.2.4 Reduced glycogen cell staining in transgenic placentae

The developmental origins of the glycogen cell are poorly understood, however an alteration to glycogen cell number is often observed in mouse models of disrupted placental development. For instance, the number of glycogen cells was increased in *Phlda2* null placentae, although the effect on glycogen cell number was not reported in the *Kvdmr1* null placenta (Frank *et al.* 2002; Salas *et al.* 2004). Glycogen cells are characterised by large glycogen-filled vacuoles and express the spongiotrophoblast marker *Tpbpa*, leading some to suggest that glycogen cells are a sub-type of the spongiotrophoblast lineage. Clusters of *Tpbpa*-positive cells were observed within the labyrinth of transgenic placentae, becoming increasingly more prominent towards term. Periodic acid Schiff staining was utilised to determine if the mislocalised *Tpbpa*-positive cells contained stored glycogen, and also to assess the relative abundance of glycogen cells in wild type and transgenic placentae.

Glycogen cells can be first identified from ~E10.5 by a small accumulation of glycogen stores, although the developmental origins of these cells appears to be ~E7.5, with expression of the glycogen cell marker *Pcdh12* first detected at this stage (Bouillot *et al.* 2005). From around E12.5, glycogen cells are identifiable by large glycogen stores and their progressive migration to the decidua (Coan *et al.* 2006). The relative abundance of glycogen cells in wild type and transgenic placentae was assessed between E10.5 and E18.5 (Fig 3.9). Few PAS-positive cells were observed in wild type and transgenic placentae at E10.5, consistent with glycogen accumulation beginning from this stage. PAS-positive staining was observed in the decidua at this stage (Fig 3.9 A and B). Glycogen staining was markedly reduced in transgenic placentae compared to wild type throughout gestation from E12.5 to E18.5 (Fig 3.9 C - J). At E16.5, glycogen cell staining of transgenic placentae appeared to persist in the spongiotrophoblast region, whereas glycogen cells of wild type placentae predominantly appeared in the maternal decidua at this stage (Fig 3.9 G and H).

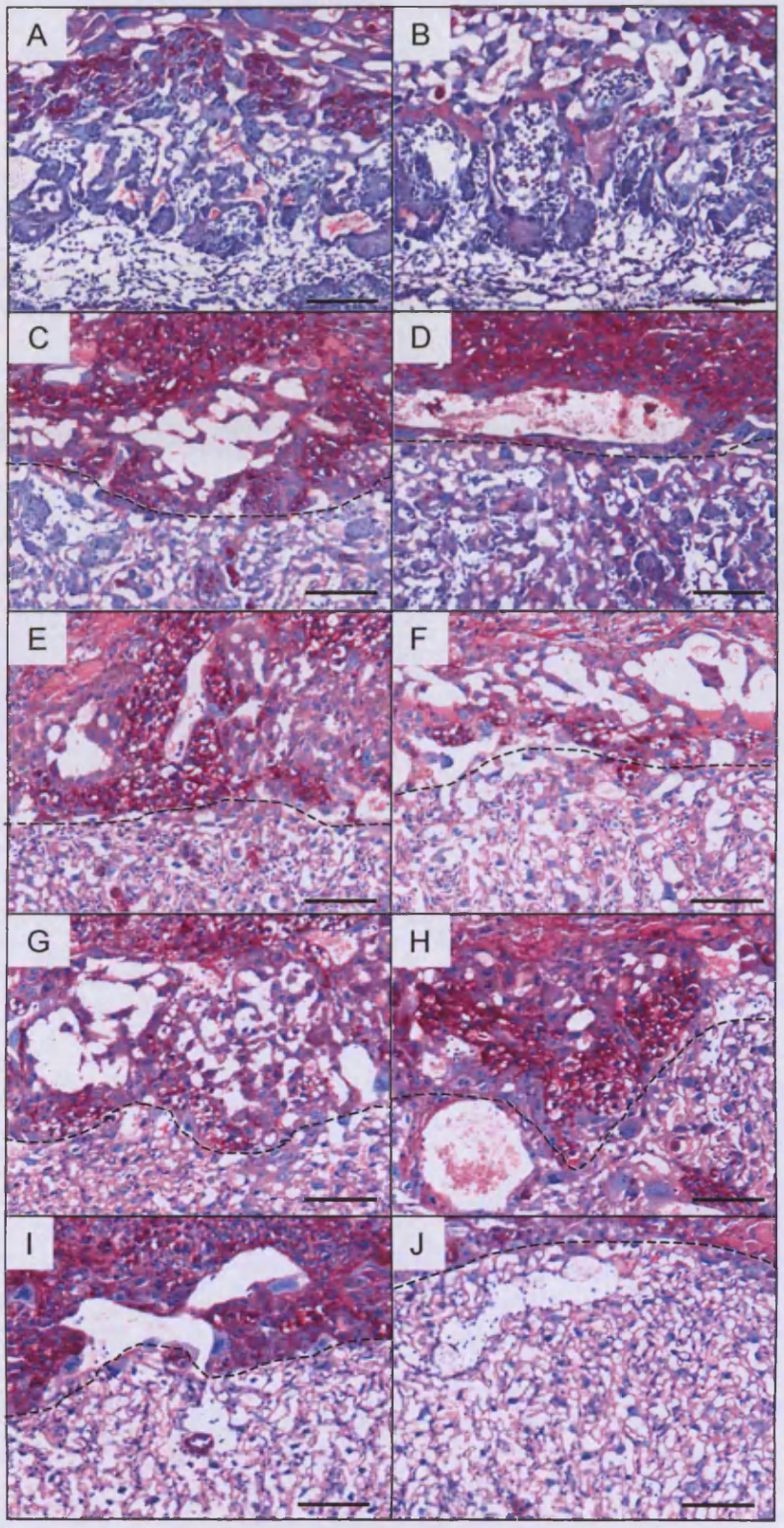


Figure 3.9: Glycogen staining as examined by Periodic acid Schiff staining

The relative abundance of glycogen cells was examined by PAS staining of midline placental sections from line 10-15 on a pure 129/Sv background. Figure shows midline placental sections of wild type (left panels) and transgenic (right panels) at (from top) E10.5, E12.5, E14.5, E16.5 and E18.5. Scale bar = 100 μm. Broken line indicates boundary between labyrinth (lower) and spongiotrophoblast (upper).

At E18.5, *Tpbpa* positive cells were mislocalised within the labyrinth of transgenic placentae (see Fig 3.8 J). Both spongiotrophoblast and the sub-population of glycogen cells express this marker, and thus to determine whether the mislocalised cells were glycogen cells, adjacent sections from E18.5 placentae were subjected to PAS staining and hybridised with the *Tpbpa* riboprobe (Fig 3.10 A - F). PAS staining was not observed to co-localise with *Tpbpa* positive cell clusters located in the labyrinth of transgenic placentae, although some occasional PAS positive cells were observed in the labyrinth of both wild type and transgenic placentae.

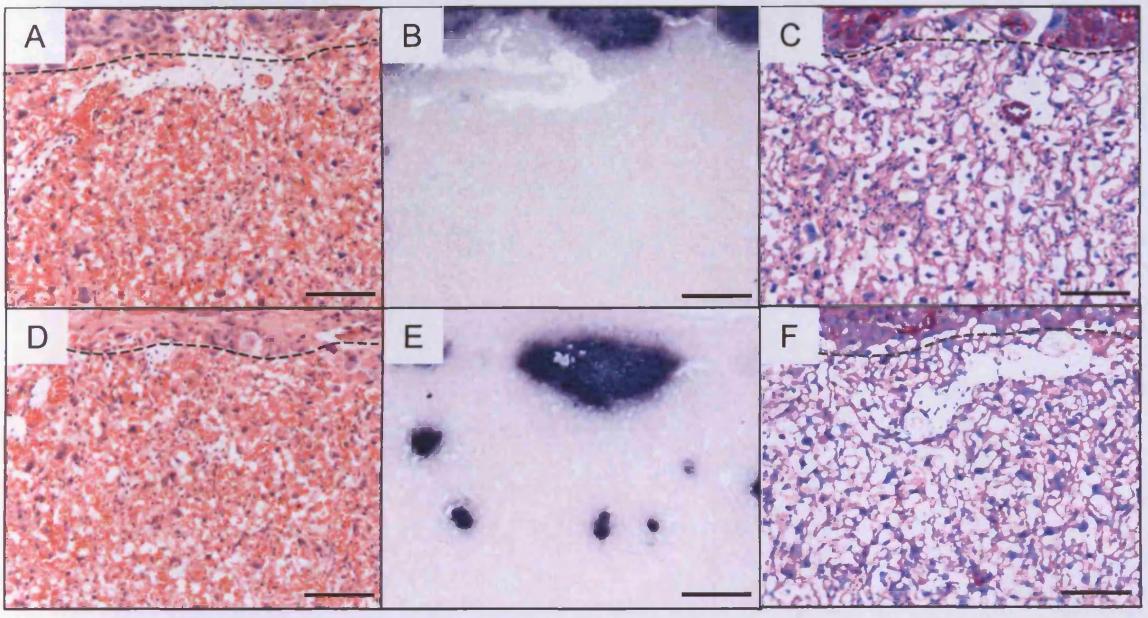


Figure 3.10: Spatial expression pattern of *Tpbpa* compared with glycogen staining at E18.5

The spatial expression of *Tpbpa* was compared with PAS staining to determine if the mislocalised *Tpbpa*-positive clusters in the labyrinth of transgenic placentae were glycogen cells. Glycogen cells were examined from serial midline placental sections of line 10-15 on a pure 129/Sv background. H&E stained sections (left panels) of wild type (upper panels) and transgenic (lower panels) show a region of labyrinth bounded by the spongiotrophoblast to the top (A and D). Adjacent sections hybridised with the *Tpbpa* riboprobe show mislocalisation in the labyrinth region of transgenic placenta (B and E). These mislocalised clusters do not co-stain for glycogen when subjected to PAS staining (C and F). Broken line indicates boundary between labyrinth (lower) and spongiotrophoblast (upper) layers. Scale bar = 100 μm.

High levels of Cdkn1c protein are reported to be a marker of glycogen cells at E15.5 (Georgiades *et al.* 2001), although at E17.5 no *Cdkn1c* mRNA was detected in glycogen cells (Takahashi *et al.* 2000). Adjacent sections of E14.5 midline placenta were stained for glycogen cells and hybridised with a *Cdkn1c* riboprobe to assess the co-localisation of staining (Fig 3.11). *Cdkn1c* mRNA appeared to co-localise, although was not specific for, glycogen cells at E14.5.

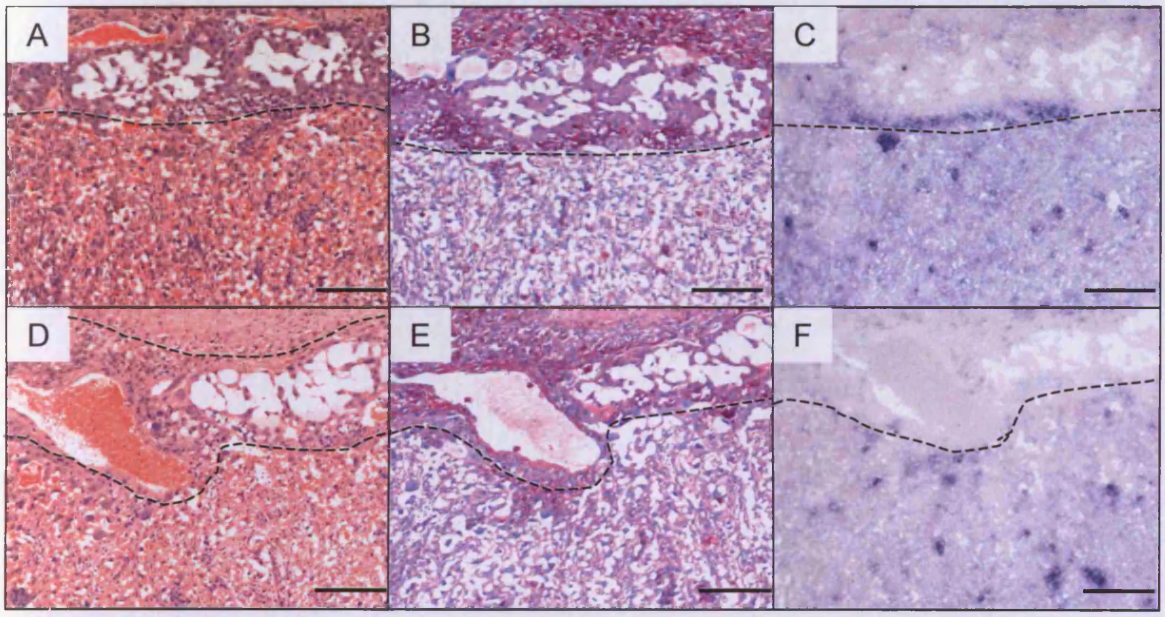


Figure 3.11: Co-localisation of *Cdkn1c* and PAS staining at E14.5

The spatial expression of *Cdkn1c* was compared with PAS staining to determine if the glycogen cells can be identified by *Cdkn1c* expression. Glycogen cells were examined from serial midline placental sections of line 10-15 on a pure 129/Sv background. H&E stained sections (left panels) of wild type (upper panels) and transgenic (lower panels) show a region of spongiotrophoblast containing glycogen cells. Adjacent sections hybridised with the *Cdkn1c* riboprobe and subjected to PAS staining show a co-localisation of positive signal. *Cdkn1c* expression was also detected in discrete cells of the labyrinth. Broken line indicates boundary between labyrinth (lower) and spongiotrophoblast (upper) layers. Scale bar = 100 μm.

3.2.6 Assessment of placental cell type marker expression

In situ hybridisation was utilised to examine expression of a number of placental gene expression markers in wild type and transgenic placenta. Several mouse models of defective placentation feature an alteration to giant cell abundance. Furthermore, there is a defined relationship between the spongiotrophoblast layer and giant cells, with giant cell precursors residing in the spongiotrophoblast layer. Although no alteration to giant cell number was reported in *Phlda2* null placentae, *Ascl2* null placentae lack spongiotrophoblast cells due to the excess differentiation of giant cells from precursors (Frank *et al.*, 2002; Guillemot *et al.*, 1994). Giant cell distribution was examined by *in situ* hybridisation of the giant cell markers *Prl3d* (formerly placental lactogen 1) and *Prl3b1* (formerly placental lactogen 2). Three placental lactogen 1 genes exist in the mouse, *placental lactogen 1 alpha* (*Prl3d1*), *beta* (*Prl3d2*) and *gamma* (*Prl3d3*), all of which are expressed only in cells of the parietal giant cell layer (Simmons *et al.* 2008). However, due to a large degree of sequence homology, the cDNA probe hybridises with all three genes, and thus expression is summarised as *Prl3d*. The placental lactogen 1 (*Prl3d*) genes exhibit transient expression between E6.5 until E11.5. Expression of *placental lactogen 2* (*Prl3b1*; formerly *Pl2*) is first detected at E11.5 and persists until term (Carney *et al.* 1993; Yamaguchi *et al.* 1992). *Prl3b1* expression is first observed in the giant cell layer only, but is later observed in all layers of the placenta (Simmons *et al.* 2007). *Prl3b1* is expressed by cells of the parietal giant cell layer in addition to the canal trophoblast giant cells and sinusoidal giant cells in the labyrinth layer. Additionally, spongiotrophoblast but not glycogen cells also express *Prl3b1* (Simmons *et al.* 2008). Accordingly, relative giant cell distribution was assessed by *in situ* hybridisation of the *Prl3d* probe at E10.5 and the *Prl3b1* probe at E12.5, E14.5 and E16.5.

There was no overt difference in *Prl3d* staining at E10.5 (Fig 3.13 A and B) with the relative distribution of *Prl3b1* staining comparable between wild type and transgenic placentae between E12.5 and E16.5 (Fig 3.13 C to H). However, the extent of *Prl3b1* staining at these stages was visibly reduced within the spongiotrophoblast region, likely representing an indirect consequence of the loss of cells from the junctional zone.

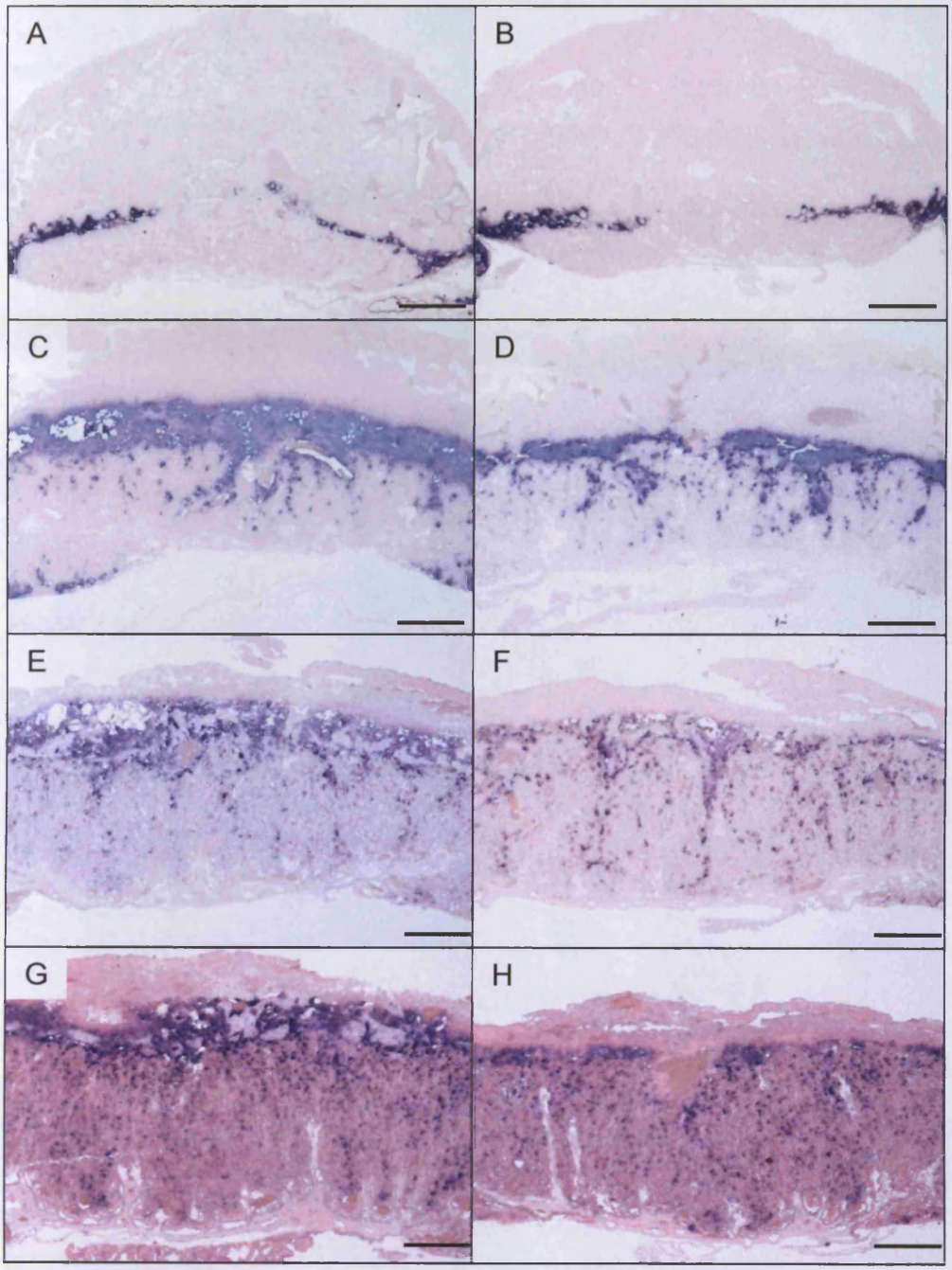


Figure 3.12: Spatial and temporal expression pattern *Prl3d* and *Prl3b1*

The relative abundance of giant cells was examined in midline placental sections from line 10-15 on a pure 129/Sv background by hybridisation with probes for *Prl3d* and *Prl3b1*. *Prl3d* expression in wild type (A) and transgenic (B) placentae at E10.5. *Prl3b1* expression in wild type (C, E, G) and transgenic (D, F, H) placentae at E12.5 (C and D), E14.5 (E and F) and E16.5 (G and H). Scale bar = 500 μm.

Ascl2 is expressed in cells of both the spongiotrophoblast and labyrinth regions with expression levels declining from E10.5 such that little *Ascl2* expression is observed at E18.5 (Rossant *et al.* 1998). At E16.5, microarray analysis on a mixed genetic background indicated a 1.7-fold lower expression level of *Ascl2*, although without achieving statistical significance. As endogenous *Ascl2* expression is relatively low at E16.5 expression was further examined by qPCR at E12.5, in addition to *in situ* hybridisation analysis of the spatial expression pattern at E10.5 and E12.5.

Expression of *Ascl2* at E12.5 was 1.5-fold (± 0.11; n = 8; $p = 0.014$) down regulated according to qPCR analysis (Fig 3.14 A). The spatial expression pattern of *Ascl2* in transgenic placentae at both E10.5 and E12.5 was largely comparable with that of wild type placentae at the same stage. The notable exception to this was the loss of *Ascl2* expression associated with spongiotrophoblast region at both stages. At E10.5, *Ascl2* expression associated with spongiotrophoblast cells was overtly reduced (Fig 3.14 C), whereas at E12.5 *Ascl2* expression was almost entirely absent from this region (Fig 3.14 E).

Ascl2 acts to maintain the spongiotrophoblast population by preventing differentiation of giant cells. The basic helix-loop-helix transcription factor encoded by *Hand1* acts antagonistically to *Ascl2* and is essential for giant cell differentiation and marks precursors of this trophoblast sub type (Riley *et al.*, 1998; Scott *et al.*, 2000). Microarray analysis of *Hand1* expression at E16.5 on a mixed genetic background identified a 1.3-fold reduction in *Hand1* expression in transgenic placentae, although without achieving statistical significance. At E12.5, expression of *Hand1* according to qPCR analysis was reduced by 1.3-fold (± 0.18; n = 8; $p = 0.014$) in transgenic placentae (Fig 3.15 A). This correlates with the reduced *Prl3b1* staining observed in the transgenic placentae and is likely an indirect consequence of the loss of spongiotrophoblast. Examination of the spatial expression pattern of *Hand1* by *in situ* hybridisation did not reveal any overt differences in *Hand1* mRNA distribution (Fig 3.15 B – E).

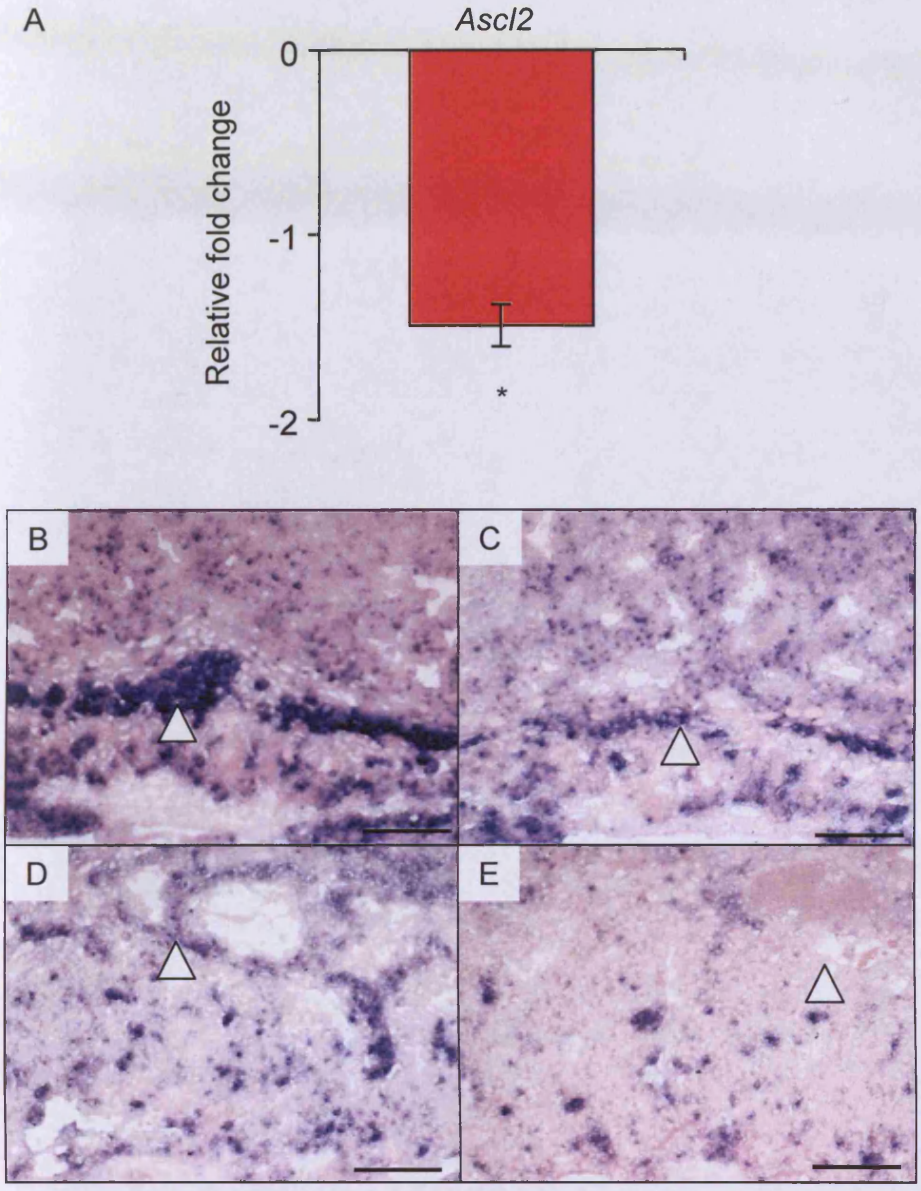


Figure 3.13: Expression of *Ascl2* at E10.5 and E12.5

Ascl2 expression was examined by qPCR at E12.5 (A) and by *in situ* hybridisation at E10.5 and E12.5 (B-E). Graph (A) shows relative fold change of *Ascl2* expression in transgenic placenta compared to wild type at E12.5. Error bar represents standard deviation. Statistical significance calculated by the Mann Whitney test is denoted by asterisks: * $p < 0.05$. *Ascl2* mRNA was examined by *in situ* hybridisation of midline placental sections from line 10-15 on a pure 129/Sv background. *Ascl2* expression at E10.5 (B and C) and E12.5 (D and E) in wild type (B and D) and transgenic (C and E) placentae. *Ascl2* expression associated with the spongiotrophoblast region was reduced or absent in transgenic placentae (arrow heads) Scale bar = 200 μm.

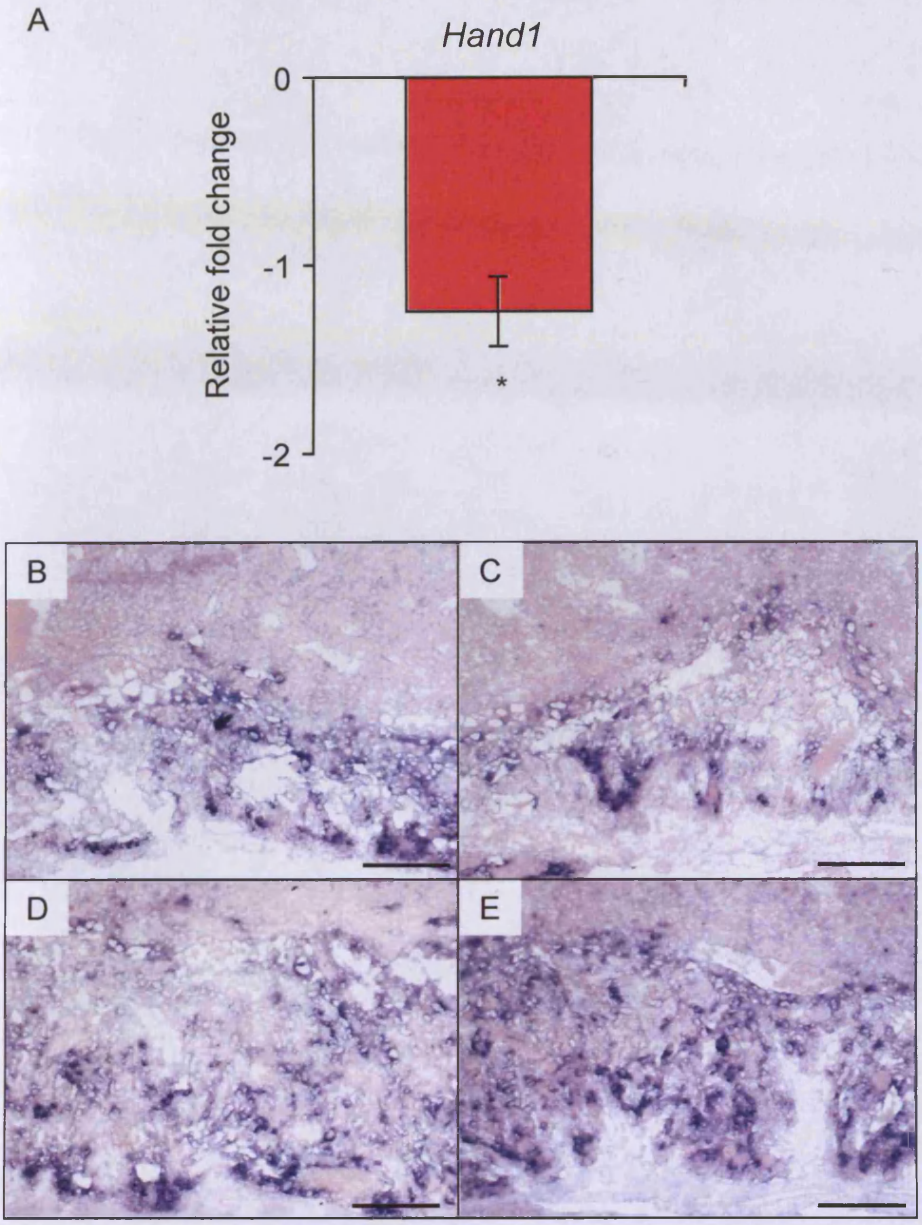


Figure 3.14: Spatial expression of *Hand1* at E10.5 and E12.5

Hand1 expression was examined by qPCR at E12.5 (A) and by *in situ* hybridisation at E10.5 and E12.5 (B-E). Graph (A) shows relative fold change of *Hand1* expression in transgenic placenta compared to wild type at E12.5. Error bar represents standard deviation. Statistical significance calculated by the Mann Whitney test is denoted by asterisks: * $p < 0.05$. *Hand1* mRNA was examined by *in situ* hybridisation of midline placental sections from line 10-15 on a pure 129/Sv background. *Hand1* expression at E10.5 (B and C) and E12.5 (D and E) in wild type (B and D) and transgenic (C and E) placentae. Scale bar = 200 μm.

Gcm1 marks branch-points of the labyrinth and is essential for branching morphogenesis of this layer of the placenta (Anson-Cartwright *et al.* 2000). Microarray analysis of *Gcm1* expression at E16.5 on a mixed genetic background indicated that there was no significant alteration to *Gcm1* expression. At E12.5, expression of *Gcm1* according to qPCR analysis was increased by 1.4-fold (± 0.16; n = 8; p = 0.014) in transgenic placentae (Fig 3.16 A). However, examination of the spatial expression pattern of *Gcm1* by *in situ* hybridisation did not reveal any overt differences in *Gcm1* mRNA distribution (Fig 3.16 B – E).

Kdr (*Flk1*) encodes the VEGF receptor-2 and marks fetal vasculature in the labyrinth layer of the placenta (Breier *et al.* 1995; Dumont *et al.* 1995). Microarray analysis of *Kdr* expression at E16.5 on a mixed genetic background revealed a statistically significant 1.5-fold elevation in expression levels in transgenic placentae. At E12.5, qPCR analysis of *Kdr* expression reported a 1.4-fold increase (± 0.31; n = 8; p = 0.014) in expression levels in transgenic placentae (Fig 3.17 A). A qualitative analysis of the spatial expression pattern of *Kdr* by *in situ* hybridisation did not reveal any overt differences in *Kdr* expression at E12.5 (Fig 3.17 B – E).

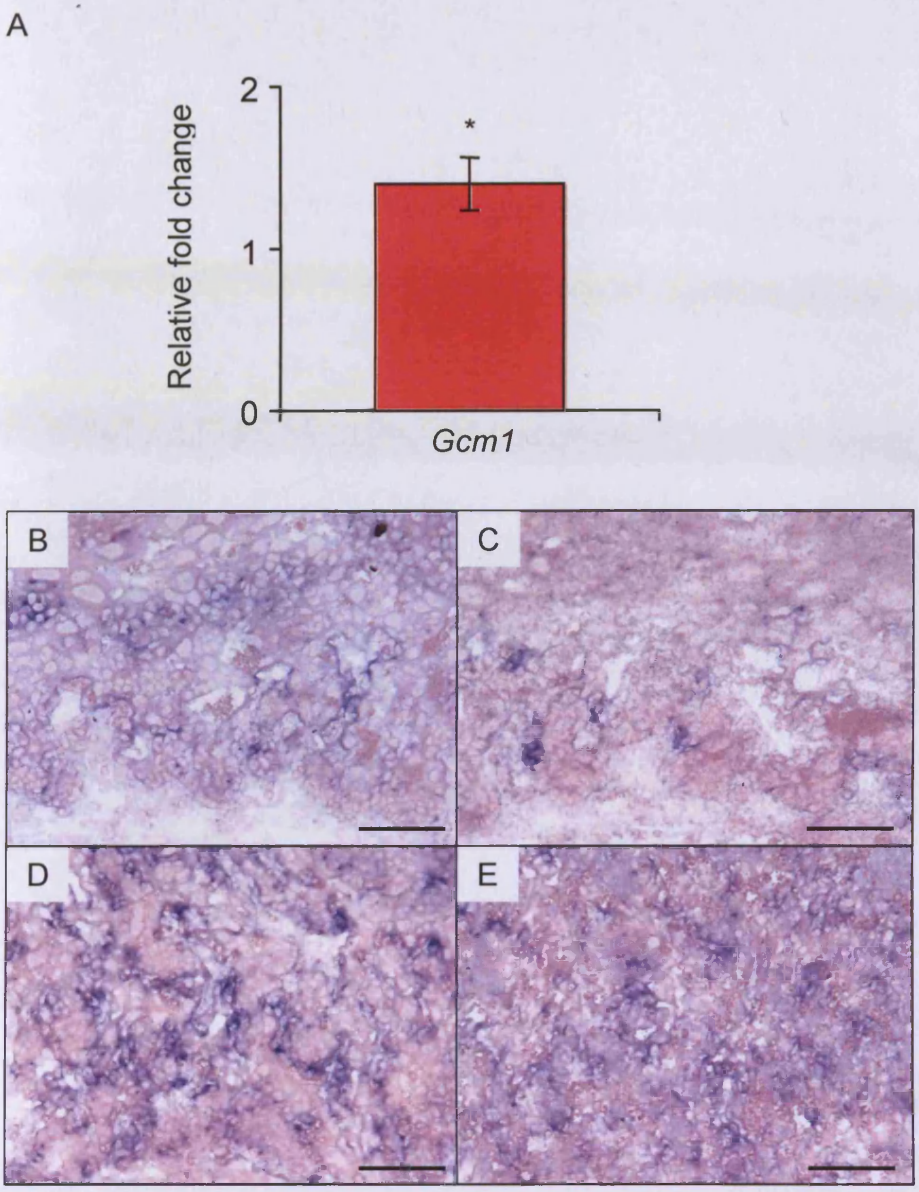


Figure 3.15: Spatial expression of *Gcm1* at E10.5 and E12.5

Gcm1 expression was examined by qPCR at E12.5 (A) and by *in situ* hybridisation at E10.5 and E12.5 (B-E). Graph (A) shows relative fold change of *Gcm1* expression in transgenic placenta compared to wild type at E12.5. Error bar represents standard deviation. Statistical significance calculated by the Mann Whitney test is denoted by asterisks: * $p < 0.05$. *Gcm1* mRNA was examined by *in situ* hybridisation of midline placental sections from line 10-15 on a pure 129/Sv background. *Gcm1* expression at E10.5 (B and C) and E12.5 (D and E) in wild type (B and D) and transgenic (C and E) placentae. Scale bar = 200 μm.

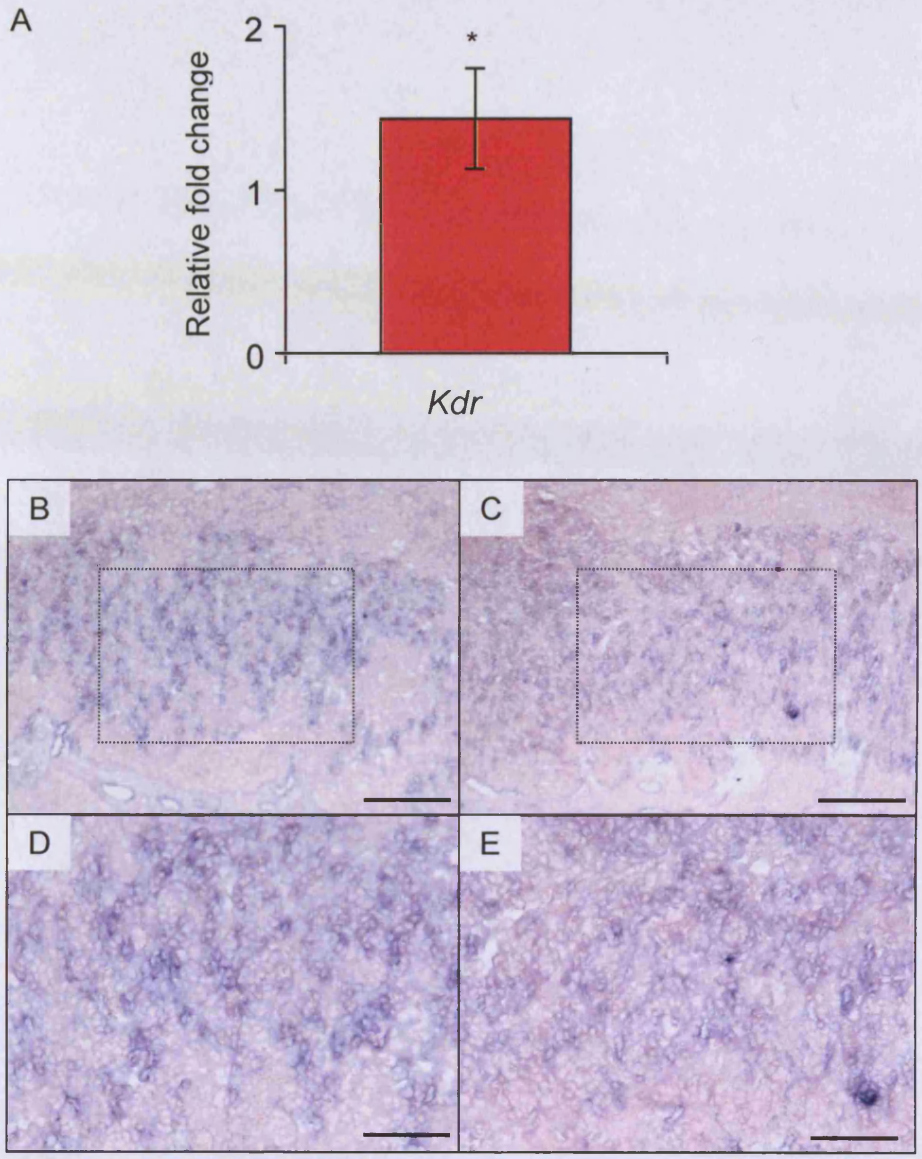


Figure 3.16: Spatial expression of *Kdr* at E12.5

Kdr (*Flk1*) expression was examined by qPCR at E12.5 (A) and by *in situ* hybridisation at E12.5. Graph (A) shows relative fold change of *Ascl2* expression in transgenic placenta compared to wild type at E12.5. Error bar represents standard deviation. Statistical significance calculated by the Mann Whitney test is denoted by asterisks: * $p < 0.05$. *Kdr* mRNA was examined by *in situ* hybridisation of midline placental sections from line 10-15 on a pure 129/Sv background. *Kdr* expression pattern under low (B and C) and higher magnification (D and E) at E12.5 in wild type (B and D) and transgenic (C and E) placentae. Scale bar = 200 μm (A and B) 100 μm (C and D).

3.3 Discussion

The principal aim of the work presented in this chapter was to further characterise the placental phenotype associated with over-expression of *Phlda2* and *Slc22a18* from a BAC transgene. Spatial and temporal expression patterns of *Phlda2* and *Slc22a18* were first examined by qPCR and *in situ* hybridisation techniques. The subsequent work builds upon initial observations reported by Salas and colleagues (2004), who reported a reduction in placental weight at E14.5 and E16.5. A more detailed examination of the placental phenotype was performed, with placental structure assessed using various techniques between E10.5 and E18.5.

3.3.1 Expression of *Phlda2* and *Slc22a18* in transgenic placenta

Expression of *Phlda2* and *Slc22a18* from the transgene as detected by Northern blotting was previously reported to be at least 10-fold greater than the level observed from the endogenous locus at E14.5 and E16.5 (Salas *et al.* 2004). In contrast, qPCR analysis of gene expression at E12.5 and E16.5 indicated that expression of these two genes was consistent with transgene copy number. This was further supported by the relative staining intensity of wild type and transgenic placental sections hybridised with probes specific for *Phlda2* or *Slc22a18*. Interestingly, *Phlda2* expression was observed at nearly 4-fold the level of wild type in *Kvdmr1*$^{+/-}$ / *Phlda2*$^{-/+}$ placentae, despite the presence of only two active copies (Salas *et al.* 2004). There are a number of potential explanations for the variable levels of expression reported for *Phlda2* and *Slc22a18* in different models. One possibility is that *Phlda2* acts via a positive feedback mechanism to directly promote its own expression. Similarly, *Phlda2* may act to maintain adjacent cells in a *Phlda2*-positive state. Although the first possibility could not be directly examined in the context of this investigation, *in situ* hybridisation of *Phlda2* mRNA at E10.5 or E12.5 did not indicate any alteration to the number of *Phlda2*-positive cells in transgenic placentae, indicating instead that elevated expression was due to increased expression in the same number of cells. Furthermore, such explanations do not address the relatively high expression of *Slc22a18*, which suggests that the observed elevated expression levels are due to over-expression of the transgene.

Another possible explanation for the variable expression levels observed is the effect of genetic background. Line 10-15 was generated by pronuclear injection of a BAC transgene containing genomic DNA from a 129/Sv genome into a C57BL/6 x CBA F1 hybrid. This line was initially bred for approximately four generations into the 129/Sv background at which point initial characterisations were performed and expression was observed at ~10-fold endogenous levels (Salas *et al.* 2004). Following a further four generations of breeding into the 129/Sv background, expression levels determined by microarray analysis revealed *Phlda2* was expressed at ~6.6-fold wild type and *Slc22a18* at ~5.4-fold wild type levels. At least a further four generations of breeding into the 129/Sv background took place prior to beginning this investigation, at which point expression levels were found to correlate with transgene copy number. The decreasing level of *Phlda2* and *Slc22a18* expression following successive generations of breeding onto the 129/Sv background demonstrates a clear effect of genetic background on transgene expression. Perhaps unsurprisingly appropriate expression of the transgene is achieved when the genetic background of the transgenic line matches the origin of the genomic fragment contained within the transgene.

Neither the spatial or temporal expression patterns of *Slc22a18* have been previously described in detail in the literature, although placental expression has been reported at E12.5 and E14.5 (Dao *et al.* 1998). *In situ* hybridisation at E12.5 revealed that *Scl22a18* is expressed by a subset of cells within the labyrinth. This expression pattern correlates with the predicted role of the *Slc22a18* gene product in solute transport. No overt difference was observed in the spatial expression pattern between wild type and transgenic placentae, with elevated expression evidently due to increased expression in a comparable number of cells. A comparison of the ΔC_T values between *Slc22a18* and the mean of the two reference genes from wild type placentae at E12.5 and E16.5 indicated an approximate 1.5-fold increase in expression level between these time-points. A similar increase in expression level was observed when ΔC_T values were compared between transgenic placentae. Thus, assuming that expression of *Gapdh* and *β-actin* remains constant between these stages, *Slc22a18* expression increases moderately between E12.5 and E16.5. A similar comparison of the ΔC_T

values for *Phlda2* revealed a ~20-fold decrease in expression between E12.5 and E16.5 in both wild type and transgenic placentae, correlating with the previously reported dramatic decline in expression from ~E12.5 (Frank *et al.* 2002).

Although no overt differences in *Phlda2* or *Slc22a18* positive cell abundance was observed between the wild type and transgenic placenta examined, cell number was not directly assessed. Ideally, the protein level of both Phlda2 and Slc22a18 could be further investigated using Western blotting to directly quantify total protein levels in wild type and transgenic placenta. Additionally, the number of cells expressing *Phlda2* and *Slc22a18* could be directly quantified from a number of placental sections to confirm that the number of cells expressing the genes is not altered. This will also enable an examination of possible ectopic expression to confirm that expression of the genes from the transgene is restricted to the cell types that endogenously express these genes.

3.3.2 Placental weight reduction of transgenic placentae

Placental weight of transgenic conceptuses was significantly reduced at all gestational stages examined between E12.5 and E18.5. The weight deficit of transgenic placentae varied between 15% and 25% from E12.5 to E16.5, with the most severe weight deficit of 25% observed at E13.5. The observed effect on placental weight was consistent with the previously reported 17% weight reduction at E14.5 and the 25% difference at E16.5 (Salas *et al.* 2004). Similarly, the degree of placental weight loss was also comparable to the extent of placentomegaly reported in *Phlda2* null mice, for which knockout placentae weighed on average 22% more than wild type at E12.5 and 27% more at E16.5 (Frank *et al.* 2002).

The extent of weight loss in transgenic placentae was greater than perhaps would have been predicted from a comparison between *Kvdmr1* null and *Kvdmr1 / Phlda2* double null mice. *Kvdmr1* null mice essentially possess one additional copy of each gene in the imprinted domain, although expression of *Phlda2* was 4-fold wild type levels. Crossing the *Kvdmr1* null line with the *Phlda2* null line generates double transgenic mice that possess one active copy of *Phlda2* but retain the two active copies of the other genes in the domain. Thus, a comparison of phenotypes between these two genotypes allowed the effect of *Phlda2* over-expression to be elucidated. Conceptuses that lack the ICR at

Kvdmr1, and thus exhibit loss of imprinting of all genes in the domain possess placentae that are ~18% lighter than wild type at both E14.5 and E16.5. Restoration of *Phlda2* gene dosage in the double transgenic placenta results in an approximate 50% rescue of the weight deficit, indicating that over-expression of *Phlda2* is associated with a ~9% reduction in placental weight. Loss of expression of *Cdkn1c* is associated with significant placentomegaly resulting from an approximate doubling of spongiotrophoblast and labyrinth cell number (Takahashi *et al.* 2000). This would suggest that over-expression of *Cdkn1c*, as occurs in the *Kvdmr1* null and *Kvdmr I Phlda2* double null placentae, would account for a significant proportion of the weight deficit observed. However, the presence of a comparable weight deficit between the BAC transgenic mice that over-express only *Phlda2* and *Slc22a18* and the *Kvdmr1* null placentae, which over-express all genes in the domain, suggest that over-expression of *Phlda2* and *Slc22a18* are the causative factors.

Alternatively, it is possible that the effect of *Phlda2* and/or *Slc22a18* over-expression is dosage dependent, with more severe phenotypes resulting from elevated expression levels. The BAC transgenic mice possess three additional copies of *Phlda2* and *Slc22a18*, whereas in *Kvdmr1* null mice, only one additional copy of each gene is present. It is also possible that over-expression of *Slc22a18* accounts for the remaining placental weight deficit in *Kvdmr1 I Phlda2* double null conceptuses. Furthermore, the genetic background of the transgenic lines examined may also be a contributory factor, with BAC transgenic mice examined on a 129/Sv background, whereas the *Kvdmr1* null mice were maintained on a 129/Sv x C57BL/6 background (Fitzpatrick *et al.* 2002) and the *Phlda2* null line on a C57BL/6 background (Frank *et al.* 2002). Each of these potential scenarios is addressed in Chapter 4.

Comparison of wild type and transgenic placental weights for both raw and normalised data sets achieved statistical significance according to the *t*-test at the 0.1% significance level for all stages examined between E12.5 and E16.5. At E18.5 however, statistical significance was only achieved when comparing normalised placental weights, with significance achieved only at the 1% significance level. Wild type placenta reached a maximum weight at E14.5, whereas the weight of transgenic placentae plateaued between E14.5 and E16.5, but subsequently increased by approximately 10% between E16.5 and E18.5.

Thus, while the difference in weight between wild type and transgenic placentae between E12.5 and E16.5 was approximately 20%, at E18.5 this weight deficit was only 10%. This late gestation increase in placental weight was only observed in transgenic placenta and may be a consequence of the reduction in spongiotrophoblast proportion at this stage. The labyrinth region continues to expand until term at the expense of the spongiotrophoblast (Coan *et al.* 2004), thus causing a relative decline in the proportion of cells that exhibit a phenotype. Alternatively, the modest increase in weight in late gestation may be compensatory for the weight deficit that has persisted throughout earlier gestation enabling increased efficiency at the time of maximal embryonic demand. The absence of a significant weight deficit at E18.5 when examining raw weight data correlates with human data in which placentae of low birth weight infants exhibited elevated *PHLDA2* expression yet did not exhibit a significant weight deficit compared to normal (Apostolidou *et al.* 2007).

3.3.3 Loss of spongiotrophoblast in transgenic placentae

The reduced placental weight observed throughout gestation was evidently due to a ~60% loss of spongiotrophoblast area at both E14.5 and E16.5. Although a slight reduction in labyrinth area was observed at both stages, this was not statistically significant, and is reciprocal to the slight expansion of the labyrinth observed in *Phlda2* null placentae (Frank *et al.* 2002). Although not quantified at earlier gestational stages, *Tpbpa* staining was overtly reduced as early as E10.5, indicating that the loss of spongiotrophoblast results from an earlier defect. The spongiotrophoblast layer comprises two distinct trophoblast sub-types, namely spongiotrophoblast cells and glycogen cells. Collectively these cell populations are sometimes referred to as the junctional zone, although this classification occasionally includes the giant cell layer in addition.

The exact role of the spongiotrophoblast layer has not been defined. However, the presence of this region is critical for embryonic survival, as exemplified by *Ascl2* null conceptuses, which have no spongiotrophoblast layer in the placenta and die at E10.5 (Guillemot *et al.* 1994). Similarly, *Birc6* deficient mice die between E11.5 and E14.5 due to loss of spongiotrophoblast (Hitz *et al.* 2005). The majority of mouse models of defective placentation exhibit perturbed labyrinth formation either in isolation or alongside other placental defects. For

example, placentomegaly associated with *Cdkn1c* null conceptuses is due to expansion of both spongiotrophoblast and labyrinth layers. Similarly, loss of placenta-specific *Igf2* expression causes a placental weight deficit due to loss of both spongiotrophoblast and labyrinth layers (Constância *et al.* 2002). Even *Ascl2* null placentae exhibit additional labyrinth layer defects and excess giant cell abundance, due to the role of Ascl2 in maintaining the spongiotrophoblast and preventing giant cell differentiation (Guillemot *et al.* 1994; Tanaka *et al.* 1997). *Phlda2* appears to be the only gene described to date that regulates development of only the junctional zone. This is perhaps even more interesting given the discrete expression of this gene in a subset of labyrinth cells.

Placentomegaly associated with *Phlda2* null placenta was due entirely to a disproportionate expansion of the spongiotrophoblast layer, including an increased abundance of glycogen cells. Although a marginal increase in labyrinth area was observed, this did not achieve statistical significance (Frank *et al.* 2002). In this investigation, transgenic placentae expressing *Phlda2* and *Slc22a18* at four-fold wild type levels displayed a weight deficit to a similar degree as the placentomegaly of *Phlda2* null conceptuses. This weight deficit could be directly attributed to a ~60% loss of spongiotrophoblast area in transgenic placenta. This was supported by a ~2-fold decrease in *Tpbpa* levels in transgenic placentae at E12.5. Similar to *Phlda2* null placentae a marginal decrease in labyrinth area was observed, although this did not achieve statistical significance. The relative distribution of glycogen cell staining was reduced throughout gestation in transgenic placenta. The gross phenotype of the BAC transgenic placentae was reciprocal to that observed in *Phlda2* null placentae. No overt labyrinth phenotype was evident, although this does not eliminate a possible phenotype at the cellular level as discussed later.

The most striking phenotype was observed at E18.5, at which stage the distinct spongiotrophoblast boundary was entirely absent from transgenic placenta, with *Tpbpa*-positive cells instead dispersed throughout the labyrinth layer. *Tpbpa* expression is specific for spongiotrophoblast and glycogen cell populations, leading some to believe that glycogen cells are a sub-type of spongiotrophoblast cells (Adamson *et al.* 2002; Georgiades *et al.* 2002). However, expression of *Protocadherin12* (*Pcdh12*), a glycogen cell specific marker (Rampon *et al.* 2005), is observed as early as E7.5, suggesting that

spongiotrophoblast and glycogen cells represent distinct cell populations (Bouillot *et al.* 2005). PAS staining was performed on adjacent sections to those hybridised with *Tpbpa* at E18.5 in an effort to determine which population of trophoblast sub-type these mislocalised clusters represented. Although PAS staining was reduced at all stages examined, the *Tpbpa*-positive clusters did not also stain with PAS, suggesting that these mislocalised cells were not glycogen cells.

Migration of glycogen cells appeared to be perturbed at E16.5 with glycogen cells of transgenic placentae observed primarily within the spongiotrophoblast layer, whereas those of wild type placenta had migrated to the decidua. Additionally, the presence of a relatively intact spongiotrophoblast layer at E16.5 followed by a complete disruption and mislocalisation of this layer at E18.5 further indicated perturbed cell migration was causative of the spongiotrophoblast mislocalisation. However, the absence of PAS staining from the *Tpbpa*-positive clusters in the labyrinth region was somewhat surprising given that spongiotrophoblast cells are not inherently a migratory cell type, whereas this is a characteristic property of glycogen cells. One possibility is that the mislocalised pockets of *Tpbpa*-positive cells represent an immature glycogen cell population. Glycogen cells can be identified from as early as E7.5 by expression of the marker *Pcdh12*, but only begin accumulating glycogen stores between E10.5 to E12.5 (Bouillot *et al.* 2005; Coan *et al.* 2006). The isolated *Tpbpa*-positive cells may thus represent this immature cell type that has failed to accumulate glycogen appropriately. Alternatively, as glycogen cell number rapidly declines between E16.5 and E18.5 due to a combination of migration and glycogen release (Coan *et al.* 2006), the mislocalised cells may represent glycogen cells that have released their glycogen stores and migrated into the labyrinth instead of the decidua.

It is interesting to note that the placentae of cloned mouse conceptuses exhibit placentomegaly characterised by an expansion of the spongiotrophoblast layer (Tanaka *et al.* 2001). For example, conceptuses generated by somatic cell nuclear transfer (SCNT) possess placenta that have an expanded spongiotrophoblast with a disrupted boundary with the labyrinth layer. Additionally, the expanded spongiotrophoblast of these placentae contain a disproportionate abundance of glycogen cells at E19.5 (Wakisaka *et al.* 2008).

Analysis of imprinted gene expression in cloned placentae revealed that expression of genes including *Igf2*, *H19* and *Cdkn1c* was appropriate. However, expression of the maternally expressed *Grb10* and paternally expressed *Mest* were reduced in cloned placentae (Inoue *et al.* 2002). Interestingly, the expanded spongiotrophoblast associated with cloned placenta correlates with the placentomegaly of *Grb10* null mice (Charalambous *et al.* 2003), although contrasts somewhat with the proportionate reduction in labyrinth and spongiotrophoblast of *Mest* deficient mice (Lefebvre *et al.* 1998). It will be interesting to determine whether expression of *Phlda2* is altered in cloned conceptuses.

Despite the absence of PAS staining in the *Tpbpa*-positive clusters in the labyrinth of transgenic placentae, it is possible that these cells indeed represent a population of glycogen cells as discussed above. Glycogen cells are characterised by a number of other markers, including *Igf2* (Redline *et al.* 1993), *Cdkn1c* (Georgiades *et al.* 2001) and *Connexin31* (*Gjb3*) (Coan *et al.* 2006). Although spongiotrophoblast cells do not express *Igf2* from mid gestation, *Igf2* expression is maintained in cells of the labyrinth layer (Carter *et al.* 2006; Redline *et al.* 1993), thus preventing glycogen cells from being specifically identified by *Igf2* expression. Furthermore, although PAS staining was observed to co-localise with *Cdkn1c* mRNA wild type and transgenic placentae at E14.5, *Cdkn1c* expression was also observed in other cells of the labyrinth and spongiotrophoblast. Furthermore, Takahashi *et al.* reported that *Cdkn1c* expression was not detectable in glycogen cells at E17.5 (Takahashi *et al.* 2000), thus eliminating *Cdkn1c* as a specific marker for glycogen cells at E18.5. *Connexin31* has recently been reported to specifically identify differentiated glycogen cells in the murine placenta (Coan *et al.* 2006). Expression of this marker may therefore be used in future to determine if the mislocalised *Tpbpa*-positive cells represent glycogen cells. Indeed, microarray analysis of gene expression revealed a significant 1.7-fold decrease in expression of the gene encoding Connexin31 (*Gjb3*).

3.3.4 Examination of placental marker expression

Expression of a number of markers representing specific trophoblast sub-types was examined by microarray and *in situ* hybridisation to determine if any subtle

placental phenotype exists that may remain undetected by histological examination of placental sections. *Ascl2* expression was significantly decreased in transgenic placenta. This decrease could be accounted for by the loss of spongiotrophoblast cells that express this gene.

A close relationship exists between spongiotrophoblast and giant cells, and thus an examination of giant cell abundance was performed by analysing expression of the giant cell markers *Prl3d* and *Prl3b1*, in addition to the transcription factor *Hand1*, which regulates giant cell differentiation. Differentiation of trophoblast cells into giant cells appears to be the default pathway (Cross 2005), although cell fate is ultimately regulated by expression of the transcription factors *Ascl2* and *Hand1*. Expression of *Hand1* marks giant cell precursors and is promotes giant cell differentiation (Scott *et al.*, 2000), whereas *Ascl2* maintains potential precursor cells in the spongiotrophoblast lineage, with loss of Ascl2 resulting in an abundance of giant cells at the expense of the spongiotrophoblast (Guillemot *et al.* 1994). At E10.5, there was no overt difference in *Prl3d* staining between wild type and transgenic placenta. At later stages, giant cell abundance was examined by expression of *Prl3b1*, the distribution of which appeared to be comparable between wild type and transgenic placenta. Reduced *Prl3b1* staining was observed in the spongiotrophoblast, consistent with endogenous expression of this gene in spongiotrophoblast cells (Simmons *et al.* 2007; Simmons *et al.* 2008), and is likely an indirect consequence of the loss of this layer in transgenic placenta.

The two placental VEGF receptors exhibit characteristic expression patterns, with *Flt1* expressed in the spongiotrophoblast and *Kdr* (*Flk1*) expressed in the labyrinth layer (Breier *et al.* 1995; Dumont *et al.* 1995). Microarray analysis of gene expression revealed a 1.7-fold decrease in *Flt1* expression, whereas *Kdr* expression was upregulated to a similar degree. Both *Flt1* and *Kdr* mark placental vasculature, with the decline in *Flt1* likely an indirect consequence of the reduced spongiotrophoblast area in transgenic placentae. *Kdr* expression level was confirmed by qPCR although *in situ* hybridisation did not reveal the cause of the apparent increase in expression. The elevated expression of *Kdr* may represent a compensatory mechanism, either in direct response to the reduced *Flt1* expression or as an indirect response to the loss of the spongiotrophoblast. It is possible that the increased *Kdr* level is indicative of increased placental

vasculature, potentially increasing the transport efficiency of the placenta. This could not be directly determined in the course of this investigation, although one approach to examine this possibility is discussed in Section 3.3.6. Alternatively, the altered expression of *Kdr* and *Flt1* may be an indirect consequence of the altered proportion of labyrinth and spongiotrophoblast cells in transgenic placenta compared to wild type.

Two striking alterations to gene expression were a ~7-fold down-regulation of *Psg18* and a ~9-fold down-regulation of *Psg19*. These pregnancy specific glycoproteins are expressed specifically by cells of the spongiotrophoblast (Kromer *et al.* 1996; Wynne *et al.* 2006), and thus the reduced expression is likely a direct consequence of the disproportionate loss of spongiotrophoblast in transgenic placentae. However, the ~7-fold reduction in transcript level does not correlate with the approximate 60% loss of spongiotrophoblast area. This suggests that the cells that secrete these glycoprotein molecules also display a disproportionate reduction in transgenic placenta. Interestingly, expression of *Cd9,* the gene encoding the Psg receptor, exhibited a moderate increase in expression according to microarray data (1.3-fold; p = 0.048). However, the altered expression of *Psg18* and *Psg19* reported from the microarray were not validated by qPCR and thus the microarray data must be interpreted cautiously. This indication of a reduction in the endocrine function of the placenta may prove useful in clarifying the function of the spongiotrophoblast layer, as discussed in Section 3.3.6.

3.3.5 Summary of main findings

The work presented in this chapter sought to build upon initial observations reported by Salas *et al.* (2004), and involved a more detailed characterisation of the placental phenotype of line 10-15 on a pure 129/Sv genetic background. Expression of both *Phlda2* and *Slc22a18* were observed to correlate with transgene copy number, in contrast to previous reports of ~10-fold endogenous levels. This over-expression appeared to be due to elevated expression in a comparable number of cells. Over-expression of the discrete locus encompassing the maternally expressed imprinted genes *Phlda2* and *Slc22a18* results in a severe placental phenotype throughout gestation:

- Placental weight deficit from at least E12.5 until term, although transgenic placenta exhibit a marginal increase in weight between E16.5 and E18.5, which was absent in wild type placenta
- Spongiotrophoblast area was reduced from at least E10.5 until term, with a reduced relative abundance of glycogen cells
- *Tpbpa*-positive cells were mislocalised within the labyrinth region at E18.5

3.3.6 Future directions

In this investigation, the placental phenotype was characterised between E10.5 and E18.5. Although placental weight could not be accurately recorded at E10.5, *in situ* hybridisation of sections at this stage clearly demonstrated a reduced expression pattern of the spongiotrophoblast marker *Tpbpa*. Future investigations could thus focus on investigating earlier stages of placental development in order to isolate the stage at which a placental phenotype is first apparent. Expression of *Phlda2* is first detectable in the polar trophectoderm, followed by the ectoplacental cone at E5.5, later becoming restricted to a subset of cells in the labyrinth layer (Frank *et al.* 1999). From mid gestation *Phlda2* expression is restricted to the syncytiotrophoblast bilayer of the labyrinth (Frank *et al.* 2002), yet the phenotypic consequences of *Phlda2* over-expression are observed on the adjacent spongiotrophoblast layer. It is possible therefore, that the spongiotrophoblast defect derives directly from altered expression of *Phlda2* in the ectoplacental cone at ~E5.5, from which the spongiotrophoblast develops. Such a phenotype would be difficult to examine *in vivo*, and thus the use of *in vitro* cell culture studies could be employed to investigate the developmental potential of various trophoblast cell types in which *Phlda2* is over-expressed.

As discussed in Section 3.3.3, the identity of the mislocalised *Tpbpa*-positive cells remains unclear. Although the absence of PAS staining indicates that these cells are not glycogen cells, the apparent migration of these cell clusters between E16.5 and E18.5 does not correlate with the properties of spongiotrophoblast cells, which are non-migratory. In contrast, glycogen cells are inherently a migratory cell type, albeit invading the maternal decidua from mid gestation. Further characterisation of these cell clusters is necessary in order to confirm which cell type they represent. The glycogen cell specific marker

Connexin31/*Gjb3* is a potential candidate by which these clusters may be identified.

Microarray analysis of the two placental VEGF receptors *Flt1* and *Kdr* (*Flk1*) revealed a striking pattern of altered expression in transgenic placentae. Expression of *Flt1*, which is restricted to the spongiotrophoblast, was significantly downregulated, a likely consequence of the disproportionate reduction in this region in transgenic placenta. In contrast, expression of the labyrinth-specific VEGF receptor *Kdr* (*Flk1*) was significantly upregulated. Expression of *Kdr* examined by *in situ* hybridisation appeared to be widespread and at a relatively low level. For this reason, it may be difficult to observe moderate differences in vasculature of the labyrinth layer. The alteration to *Kdr* expression level may represent a compensatory mechanism of the placenta of increasing fetal blood flow to minimise the impact of the reduced spongiotrophoblast layer on embryonic growth. A direct method of examining placental vasculature would be to generate resin vascular casts of wild type and transgenic placenta as described by Adamson *et al.* (2002). Vasculature could be examined at various stages throughout gestation. If elevated *Kdr* represents an increase in vasculature of the labyrinth as a response to the reduced spongiotrophoblast, then this could be directly quantified from the vascular casts. An alternative approach to creating vascular casts would be to use stereology examine the structure of the labyrinth layer. This would enable a direct quantification of all volume, surface area and length measurements in this region of the placenta as described by Coan *et al.* (2004).

Further work may also be directed to further investigating the altered expression of the *Psg18* and *Psg19* genes reported on the microarray. Initially, these altered expression levels must be validated by qPCR, with expression levels of additional *Psg* genes also potential targets of qPCR analysis. Such dramatically reduced levels of pregnancy-related hormones may be indicative of the functional role of the spongiotrophoblast, which to date has not been definitively characterised. The spongiotrophoblast is predicted to perform an endocrine role through secreting hormones that target maternal physiology to favour embryonic growth. However, a definitive role for this region of the mouse placenta has not been described to date. The transgenic model described in this chapter is unique in only exhibiting a spongiotrophoblast defect, and may thus

provide an invaluable tool for elucidating the function of the spongiotrophoblast. If the spongiotrophoblast secretes hormones that alter maternal physiology, this could be investigated in pregnant females containing conceptuses that are all hemizygous for the BAC transgene described previously. All such conceptuses would have placentae with a significantly reduced spongiotrophoblast layer. This would be predicted to result in a reduction in the level of circulating hormones in the pregnant dam. These levels could be compared against those of pregnant females containing entirely wild type conceptuses in a similar sized litter.

The placental phenotype described in this chapter correlates with the initial reports of a placental weight deficit at E14.5 and E16.5. *Phlda2* was isolated as the likely cause of this phenotype from examination of a *Kvdmr1* / *Phlda2* double null placenta. The weight deficit was additionally attributed to a disproportionate loss of the spongiotrophoblast (Salas *et al.* 2004). Although evidence suggests a causative role for *Phlda2* in the phenotype, the presence of *Slc22a18* on the transgene means that a role for this gene cannot be ruled out. This scenario is addressed in Chapter 4.

Chapter 4:

Effect of gene dosage and genetic background on placental phenotype

4.1 Overview

The effect of over-expressing a discrete imprinted locus containing the maternally expressed genes *Phlda2* and *Slc22a18* was discussed in Chapter 3. These characterisations were performed using mice on a pure 129/Sv genetic background. An important aspect to consider when investigating mouse models that possess a transgene is the indirect effect that integration of this transgene may have. For instance, the transgene may integrate within the coding region of another gene. Any observed phenotype may therefore be the result of either excess expression from the transgene or loss of expression of the gene at the site of integration. In order to eliminate the possibility that the placental phenotype is a consequence of the site of transgene integration, rather than over-expression of the genes *Phlda2* and/or *Slc22a18*, an independent transgenic line was generated containing the same modified BAC transgene.

This second transgenic line was named 10-10, and was initially maintained on a C57BL/6 background, with phenotypic analysis performed on the 129/Sv x C57BL/6 background. To enable a direct comparison between the transgenic lines 10-15 and 10-10, line 10-15 was also characterised on the 129/Sv x C57BL/6 background. The effect of genetic background of mouse models is often not a major consideration when performing phenotypic analyses. There is, however precedent that genetic background affects the phenotype of mouse models of placentation. For instance, the degree of placentomegaly in *Phlda2* null animals at E16.5 was increased in a pure C57BL/6 background (33% larger than wild type) compared with the C57BL/6 x 129/Sv background (27% than wild type) (Frank *et al.* 2002).

A genetic background-dependent placental defect is also observed in Myod family inhibitor *Mdfi* (*I-mfa*) null mice. Homozygous null mice on a C57BL/6 background have significantly fewer trophoblast giant cells with embryonic death resulting at E10.5. In contrast, the same mutation on the 129/Sv background does not cause a placental phenotype although embryos display neurological defects (Kraut *et al.* 1998). Additionally, mice that lack expression of the gene encoding the epidermal growth factor receptor (*Egfr*) have smaller placentae with a reduced spongiotrophoblast layer. On a pure 129/Sv background, embryonic

death occurs around E12.5, whereas some embryos survive to birth on the 129/Sv x C57BL/6 background (Sibilia and Wagner 1995; Threadgill *et al.* 1995).

The placental phenotype was also examined in an additional two transgenic lines, which possess either one or two copies of the unmodified transgene. These animals contain intact copies of *Cdkn1c* on the transgene. However, placental-specific enhancer elements are absent and thus *Cdkn1c* is only over-expressed in a subset of embryonic tissues (John *et al.* 2001). Use of these transgenic lines, in addition to line 10-10, enabled an examination of the effect of transgene copy number on the placental phenotype. More importantly, line 5D3, which contains a single copy of the unmodified transgene, represents a model for LOI at the discrete locus containing the imprinted genes *Phlda2* and *Slc22a18*. The placental phenotype observed in lines 10-15 and 10-10 was therefore compared with that of line 5D3 to determine whether the multiple copy transgenic lines exhibit a similar phenotype.

To elucidate the relative contribution of *Phlda2* and *Slc22a18* to the observed placental phenotype, the placental phenotype was examined in placentae in which *Phlda2* gene dosage was restored to normal while *Slc22a18* remains elevated. Such "double transgenic" conceptuses were generated by mating a hemizygous 5D3 transgenic male with a heterozygous *Phlda2* null female. A direct comparison of double transgenic placentae with both wild type and 5D3 transgenic placentae from the same litter was undertaken to examine the effect of restoring *Phlda2* gene dosage to normal. A similar approach was undertaken to investigate over-expression of *Phlda2* by comparing *Kvdmr1* null placentae and double *Kvdmr1* and *Phlda2* null placentae (Salas *et al.* 2004).

4.2 Results

4.2.1 Characterising line 10-15 on the 129/Sv x C57BL/6 background

The effect of over-expressing *Phlda2* and *Slc22a18* was initially examined using mice on a pure 129/Sv genetic background (Chapter 3). The placental phenotype of line 10-15 was additionally investigated on the 129/Sv x C57BL/6 genetic background to determine if there were any differences in the phenotype. This was necessary, as further analysis of the placental phenotype would include three independent lines maintained on a different genetic background.

Expression levels of *Phlda2* and *Slc22a18* were consistent with transgene copy number in line 10-15 on a pure 129/Sv background (see Section 3.2.1). To confirm that similar expression levels were maintained on the 129/Sv x C57BL/6 background the expression of *Phlda2* and *Slc22a18* was assessed by qPCR at E12.5. *Phlda2* was expressed at 3.0-fold (± 0.18) wild type and *Slc22a18* was expressed at 3.78-fold (± 1.39) wild type levels (Fig 4.1).

On a pure 129/Sv background, mean transgenic placental weight was ~15% less than that of wild type at E14.5, with an approximate 50% compensation in placental weight occurring by E18.5, with transgenic placentae ~9% lighter. Placental weight was examined at E14.5 and E18.5 on the 129/Sv x C57BL/6 background, with data presented in Figure 4.2. At E14.5, a similar weight difference to transgenic placenta on a pure 129/Sv background was observed, with transgenic placentae 19.1% lighter than wild type (104.7 mg ± 8.4 versus 84.7 mg ± 9.0; 5 litters; n = 35; $p = 1.07 \times 10^{-7}$). In contrast to transgenic placenta on a 129/Sv background, this degree of weight deficit persisted to E18.5, with transgenic placentae 19.6% lighter at this stage (118.9 mg ± 6.2 versus 95.6 mg ± 8.2; 6 litters; n = 37; $p = 2.22 \times 10^{-11}$).

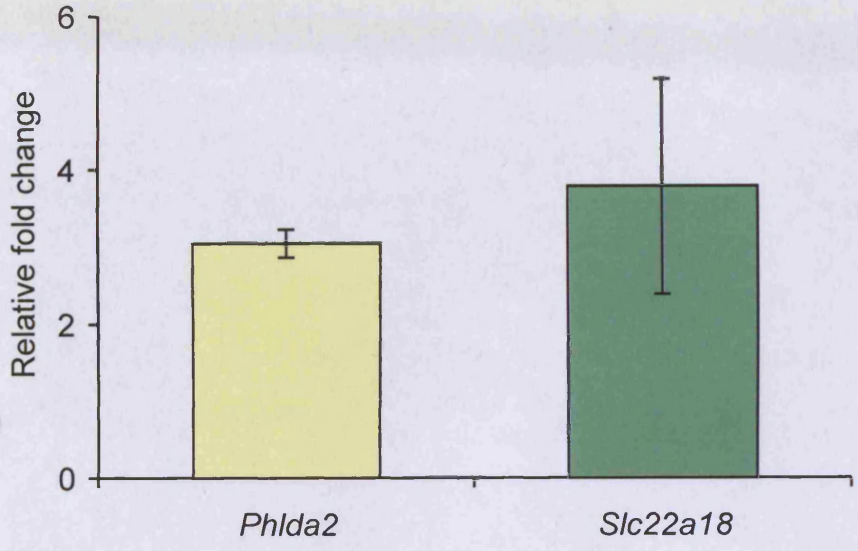


Figure 4.1: Transgene expression at E12.5 in placentae of line 10-15 on the 129/Sv x C57BL/6 background

Expression of *Phlda2* and *Slc22a18* was examined in E12.5 placentae from line 10-15 on the 129/Sv x C57BL/6 background to determine if appropriate expression levels were maintained. Graph shows mean fold change for *Phlda2* and *Slc22a18* in transgenic placentae compared to wild type placentae from the same litter. Error bars represent standard deviation between independent samples.

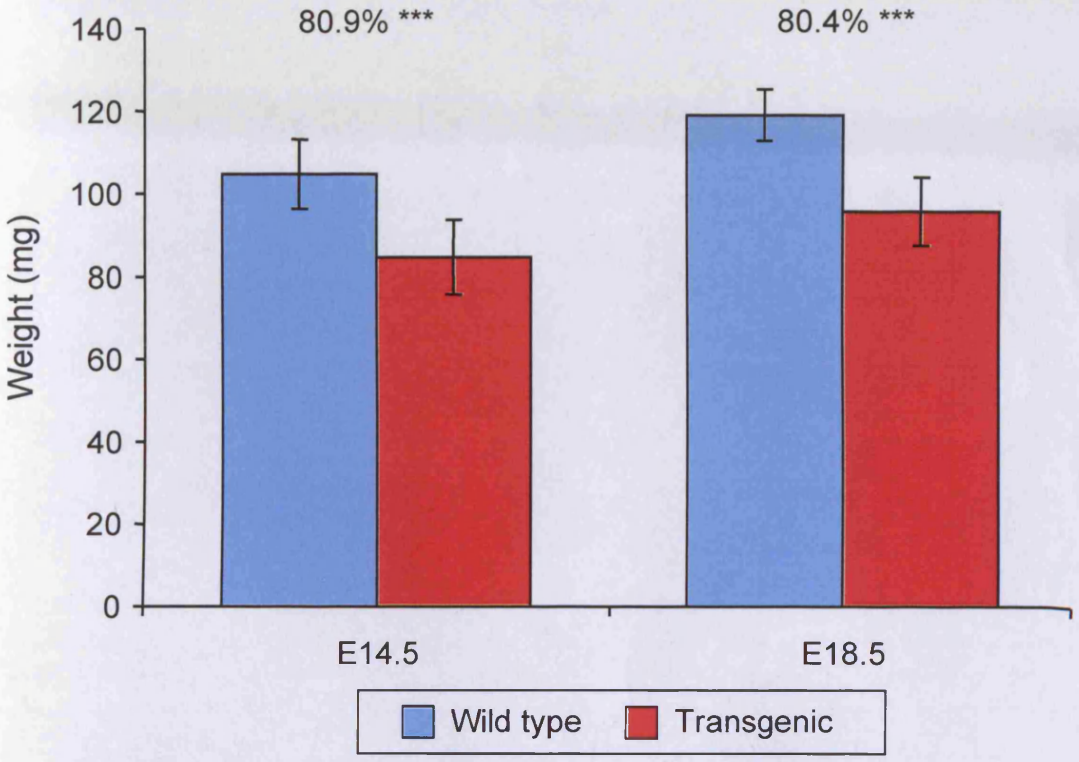


Figure 4.2: Placental weights at E14.5 and E18.5 from line 10-15 on the 129/Sv x C57BL/6 background

To confirm that the placental phenotype persists on the 129/Sv x C57BL/6 background, placental weights were examined at E14.5 and E18.5. The graph shows the mean placental weight of wild type and transgenic placentae, with error bars representing standard deviation. Percentage values indicate the ratio of mean transgenic weight to wild type. Statistical significance as determined by *t*-test is denoted by asterisks *** $p < 0.001$.

Transgenic placentae of line 10-15 on a pure 129/Sv background exhibit a significantly reduced spongiotrophoblast area at E14.5, with a complete loss of the spongiotrophoblast-labyrinth boundary and mislocalisation of *Tpbpa*-positive cells in the labyrinth at E18.5. E14.5 and E18.5 placentae from line 10-15 on the 129/Sv x C57BL/6 background were hybridised with the *Tpbpa* probe to examine the extent of spongiotrophoblast loss and mislocalisation (Fig 4.3). At both E14.5 and E18.5, the spongiotrophoblast was overtly reduced in size in transgenic placenta, with *Tpbpa*-positive cells observed in the labyrinth of transgenic placentae at both stages. Some *Tpbpa*-positive cells were also detected in the labyrinth of wild type placentae at E14.5, although to a lesser extent compared to transgenic, and with no such mislocalisation in wild type placentae at E18.5. At E18.5, transgenic placentae exhibit a similar pattern of *Tpbpa*-positive cells in the labyrinth, although not to the extent that is seen on the pure 129/Sv background, with a relatively intact spongiotrophoblast-labyrinth border existing at this stage (Fig 4.3F and H)

The area of placental regions was measured as before at E14.5. Spongiotrophoblast area was significantly reduced in transgenic placentae, accounting for only 57% of the area in wild type placentae (3.17 mm^2 ± 0.76 versus 1.81 mm^2 ± 0.42; 4 litters; n = 22; $p = 4.19 \times 10^{-4}$) (Fig 4.4). No significant effect on labyrinth area was observed (4.02 mm^2 ± 0.56 versus 3.78 mm^2 ± 0.60; $p = 0.339$), with the combined area of the two regions reduced by approximately 21% (7.16 mm^2 ± 0.74 versus 5.63 mm^2 ± 0.82; $p = 6.84 \times 10^{-4}$).

To examine the glycogen content of wild type and transgenic placentae, PAS staining was performed at E14.5 and E18.5. Similar to transgenic placentae on the pure 129/Sv background, PAS staining was markedly reduced in transgenic placentae on the 129/Sv x C57BL/6 background at both stages (Fig 4.5 C, D, G and H). Occasional PAS-positive staining was observed in the labyrinth, however this was seen in both wild type and transgenic placentae and did not consistently co-localise with *Tpbpa*-positive cells within the labyrinth.

In summary, the placental phenotype of line 10-15 on a pure 129/Sv background was maintained on a 129/Sv x C57BL/6 background. Although there were two notable variations, with the ~20% placental weight deficit persisting to a similar degree at E18.5 on the 129/Sv x C57BL/6 background, at which stage the spongiotrophoblast mislocalisation defect appeared to be less severe.

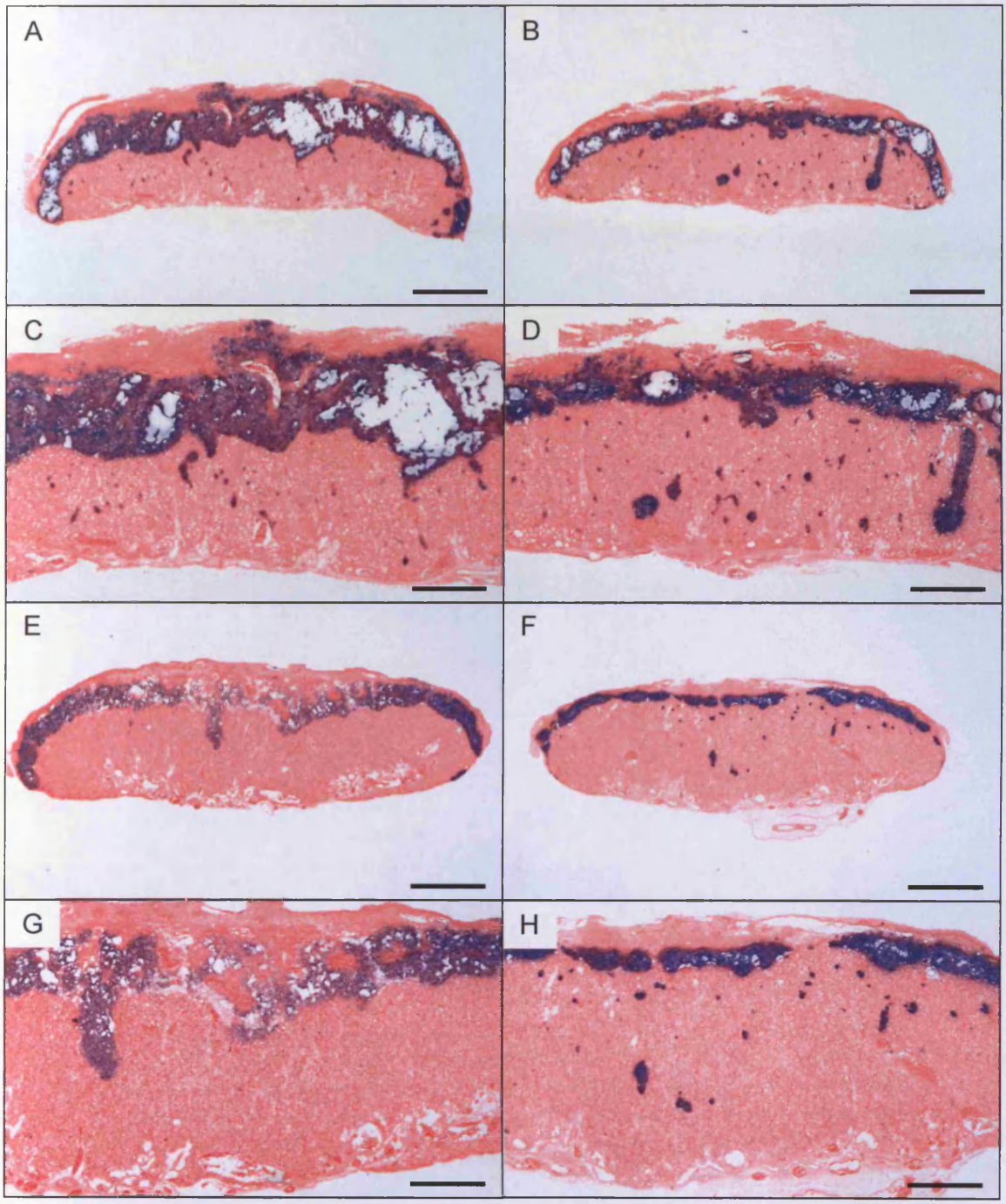


Figure 4.3: Loss of spongiotrophoblast in line 10-15 on the 129/Sv x C57BL/6 background

To visualise placental structure and determine if spongiotrophoblast was reduced in transgenic placentae, placental sections were hybridised with the *Tpbpa* riboprobe. Wild type sections (left column) and transgenic sections (right column) were examined at E14.5 (A-D) and E18.5 (E-H). Sections in A, B, E and F are shown under higher magnification in C, D, G and H respectively. Scale bar = 1 mm (A, B, E, F); and 0.5 mm (C, D, G, H).

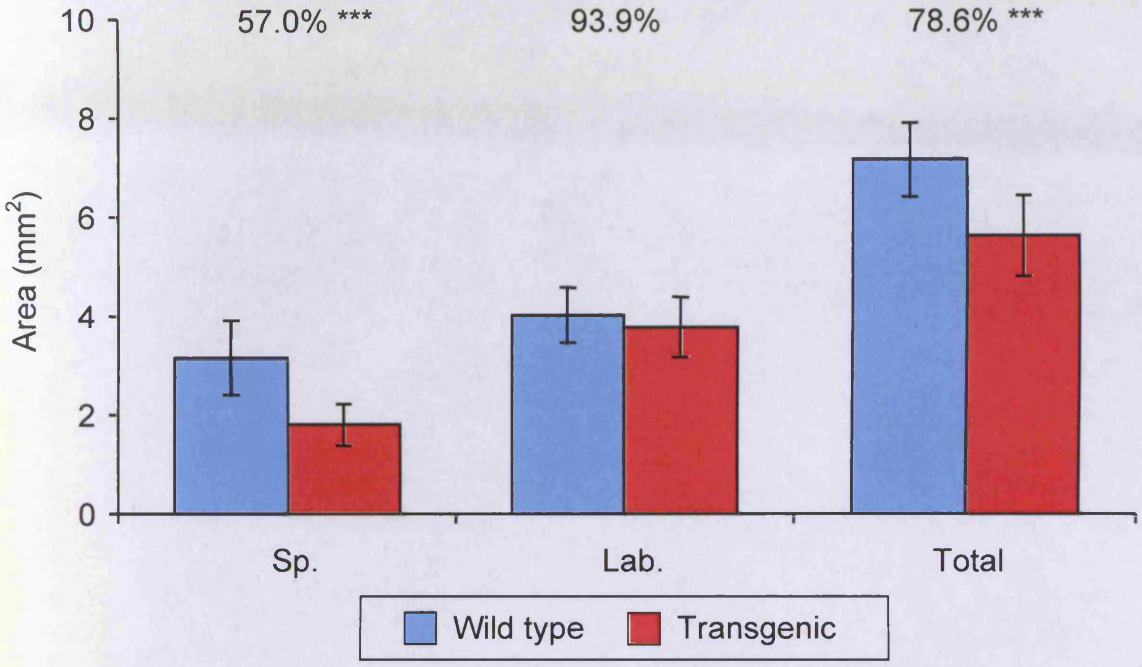


Figure 4.4: Midline placental areas at E14.5 from line 10-15 on the 129/Sv x C57BL/6 background

Spongiotrophoblast and labyrinth regions were measured at E14.5 to determine the extent of spongiotrophoblast loss. Graph shows mean area of spongiotrophoblast (Sp.), labyrinth (Lab.) and combined spongiotrophoblast and labyrinth regions (Total), with error bars representing standard deviation. Percentage values show the ratio of transgenic to wild type area. Statistical significance as determined by Mann-Whitney test is denoted by asterisks; *** $p < 0.05$.

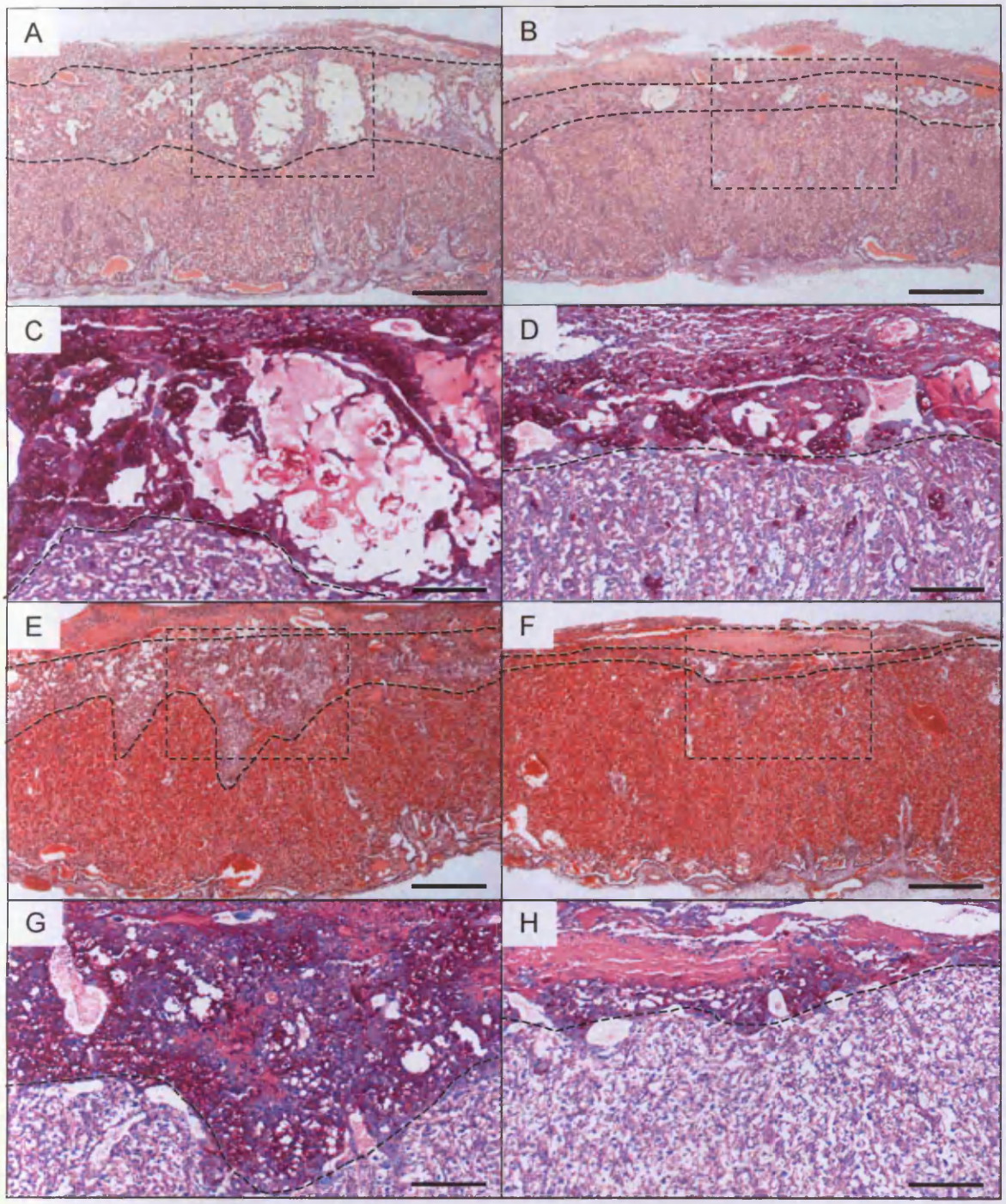


Figure 4.5: Glycogen cells in placentae of line 10-15 on the 129/Sv x C57BL/6 background

General placental morphology was examined by H&E staining, with relative glycogen cell number assessed by PAS staining. Figure shows H&E (A, B, E, F) and PAS (C, D, G, H) staining at E14.5 (upper four) and E18.5 (lower four) of wild type (left column) and transgenic (right column) placentae. Dashed boxes indicate region of section stained for glycogen cells and shown at higher magnification below. Broken lines indicate boundaries of placental layers; labyrinth at bottom. Scale bar = 500 μm (H&E) and 200 μm (PAS).

4.2.2 Characterising line 10-10 on the 129/Sv x C57BL/6 background

A second transgenic line was also generated that possesses the same modified transgene as line 10-15 but at an independent integration site and with an unconfirmed copy number. This line was generated by pronuclear injection into C57BL/6 x CBA F_1 embryos and subsequently bred into the C57BL/6 background. The placental phenotype was studied on a 129/Sv x C57BL/6 background thus allowing a direct comparison with placentae from line 10-15 on a similar genetic background.

The transgene copy number of line 10-10 was not quantified by Southern blotting. However, expression of *Phlda2* and *Slc22a18* was examined at by qPCR E12.5 from placenta on the 129/Sv x C57BL/6 background. *Phlda2* was expressed at 2.8-fold (± 0.38) wild type and *Slc22a18* at 1.8-fold (± 0.56) wild type level (Fig 4.6), suggesting that the line contained 1-2 copies of the BAC transgene.

Transgenic placentae from line 10-15 on the 129/Sv x C57BL/6 background were approximately 20% lighter than wild type at both E14.5 and E18.5 (see Section 4.2.1). A similar effect on placental weight was observed in line 10-10, with transgenic placentae 20.2% lighter than wild type at E14.5 (111.0 mg ± 9.0 versus 88.6 mg ± 9.8; 7 litters; n = 46; $p = 3.12 \times 10^{-10}$), and 16.9% lighter at E18.5 (112.3 mg ± 6.8 versus 93.4 mg ± 9.3; 5 litters; n = 34; $p = 1.01 \times 10^{-7}$) (Fig 4.7).

Placentae from line 10-10 at E14.5 and E18.5 were hybridised with the *Tpbpa* probe to examine the extent of spongiotrophoblast loss and mislocalisation (Fig 4.8). At both E14.5 and E18.5, the spongiotrophoblast was overtly reduced in size in transgenic placenta. Although discrete clusters of *Tpbpa*-positive cells were observed in the labyrinth of transgenic placentae at both stages, this feature of the phenotype was not as pronounced at E18.5 as for line 10-15 on both genetic backgrounds examined at this stage.

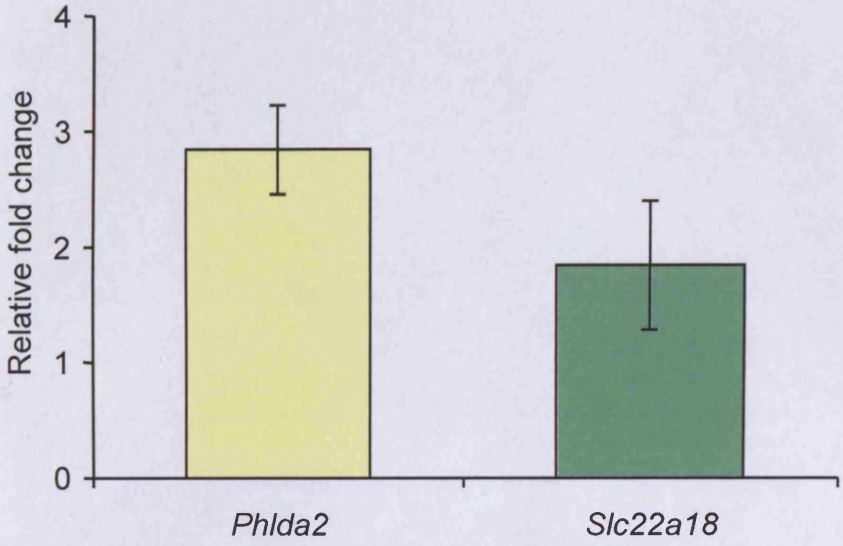


Figure 4.6: Transgene expression at E12.5 in placentae of line 10-10 on the 129/Sv x C57BL/6 background

Expression of *Phlda2* and *Slc22a18* was examined at E12.5 in placentae from line 10-10 on the 129/Sv x C57BL/6 background to estimate the transgene copy number and allow a comparison with line 10-15 for which transgene copy number was known. Graph shows mean fold change for *Phlda2* and *Slc22a18* in transgenic placentae compared to wild type placentae from the same litter. Error bars show standard deviation.

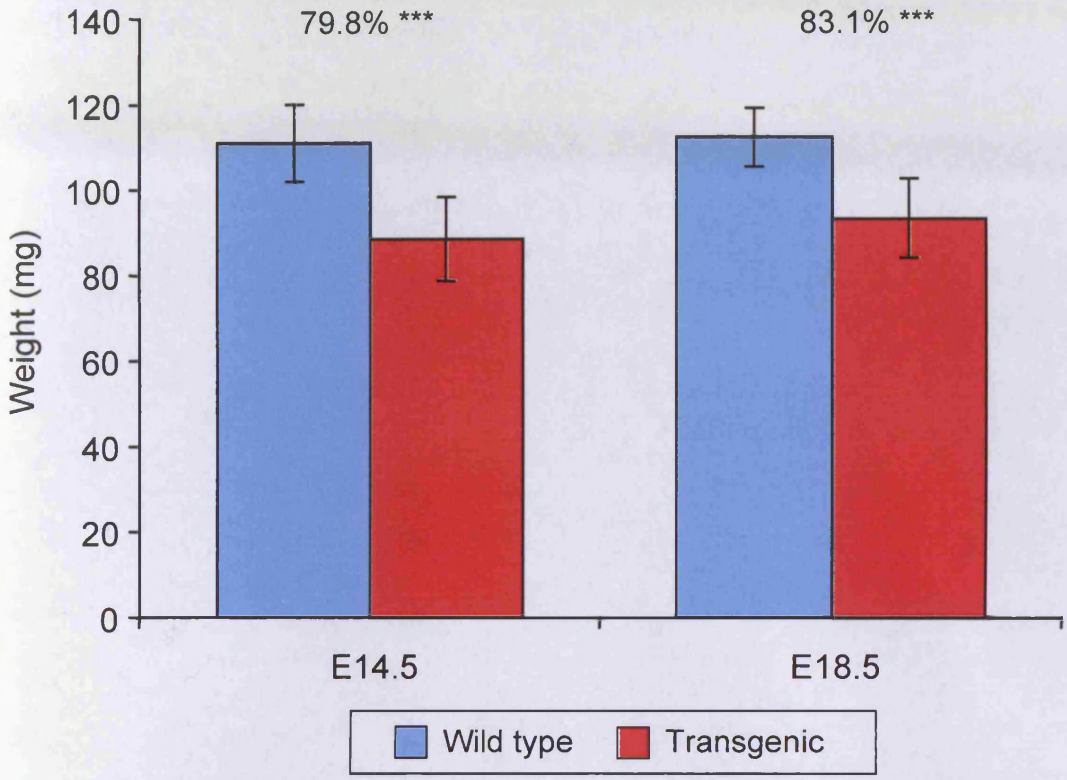


Figure 4.7: Placental weights at E14.5 and E18.5 from line 10-10 on the 129/Sv x C57BL/6 background

To confirm that the placental phenotype is due to over-expression of the imprinted locus, placental weights were examined at E14.5 and E18.5 from the independent line 10-10. The graph shows the mean placental weight of wild type and transgenic placentae, with error bars representing standard deviation. Percentage values indicate the ratio of transgenic weight to wild type. Statistical significance as determined by *t*-test is denoted by asterisks *** $p < 0.001$.

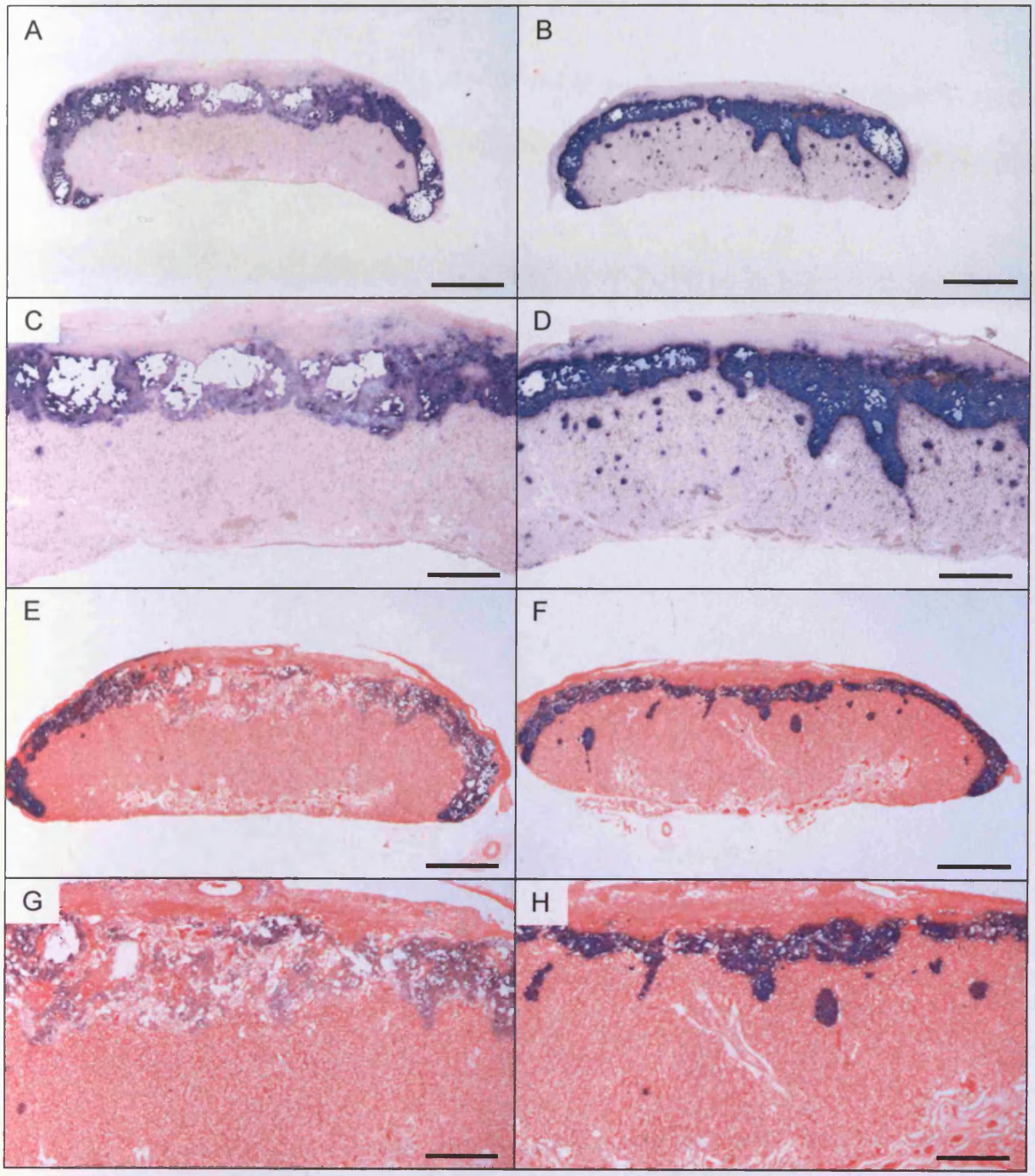


Figure 4.8: Loss of spongiotrophoblast in line 10-10 on the 129/Sv x C57BL/6 background

To visualise placental structure and determine if spongiotrophoblast was reduced in transgenic placentae, placental sections from line 10-10 were hybridised with the *Tpbpa* riboprobe. Wild type sections (left column) and transgenic sections (right column) were examined at E14.5 (A-D) and E18.5 (E-H). Sections in A, B, E and F are shown under higher magnification in C, D, G and H respectively. Scale bar = 1 mm (A, B, E, F); and 0.5 mm (C, D, G, H).

At E14.5, similar to line 10-15 on both a pure 129/Sv and 129/Sv x C57BL/6 background, the spongiotrophoblast area was significantly reduced in transgenic placentae of line 10-10, accounting for only 54.9% the area in wild type placentae (3.17 mm^2 ± 0.54 versus 1.74 mm^2 ± 0.49; 4 litters; n = 29; p = 2.10 x 10^{-5}). No significant effect on labyrinth area was observed (3.75 mm^2 ± 0.91 versus 3.39 mm^2 ± 1.19; p = 0.511), with the combined area of the two regions reduced by approximately 25% (6.91 mm^2 ± 1.20 versus 5.17 mm^2 ± 1.46; p = 3.30 x 10^{-3}) (Fig 4.9).

Glycogen cell staining was overtly reduced in transgenic placentae at both stages examined (Fig 4.10). Although some glycogen cells were detected in the labyrinth region of transgenic placentae at E14.5, this did not correlate with, the number of *Tpbpa*-positive clusters in the transgenic placentae.

In summary, the placental phenotype of line 10-10 on a 129/Sv x C57BL/6 genetic background was comparable to that of line 10-15 on a similar genetic background. Similar to line 10-15 on the 129/Sv x C57BL/6 background, the placental weight deficit observed at E14.5 persisted to a similar degree at E18.5, with a similar mislocalisation phenotype apparent at this stage. This comparable placental phenotype is despite an apparently lower expression of *Phlda2* and *Slc22a18* in transgenic placentae of line 10-10.

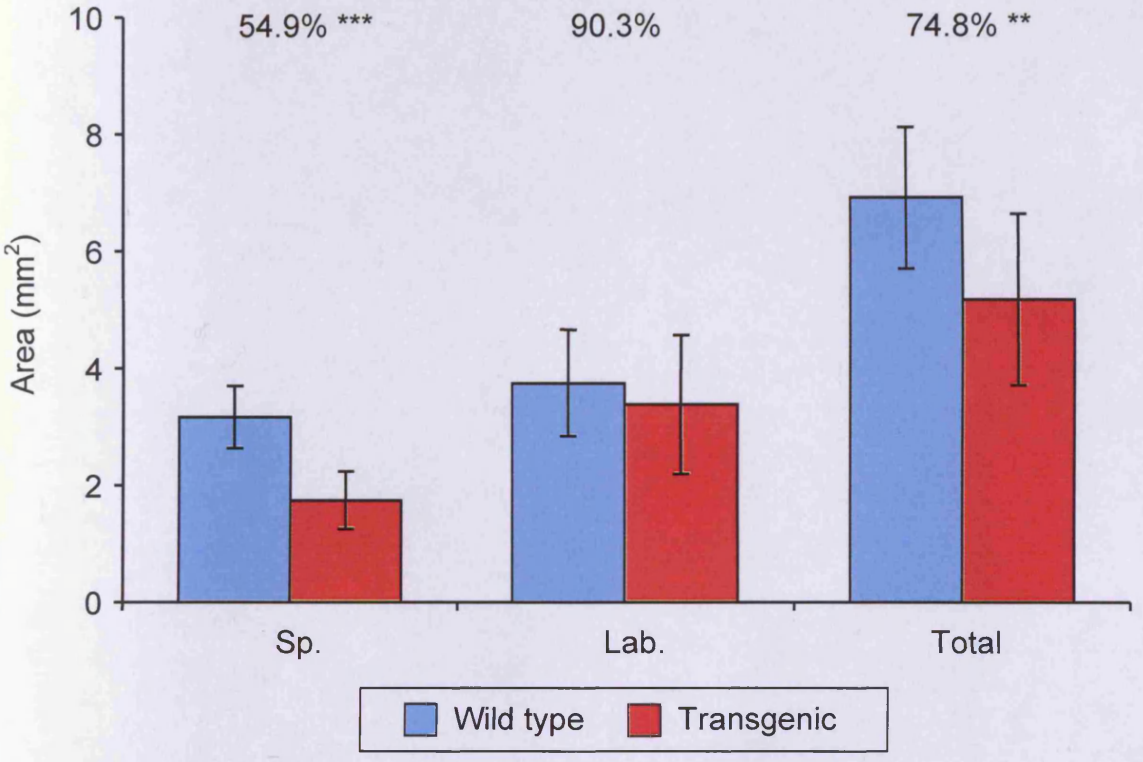


Figure 4.9: Midline placental areas at E14.5 from line 10-10 on the 129/Sv x C57BL/6 background

Areas of spongiotrophoblast, labyrinth and combined areas (total) from midline placental sections at E14.5 from line 10-10. Graph shows mean midline areas of spongiotrophoblast (Sp.), labyrinth (Lab.) and combined spongiotrophoblast and labyrinth regions (Total), with error bars representing standard deviation. Percentage values indicate the ratio of transgenic area to wild type. Statistical significance as determined by Mann-Whitney test is denoted by asterisks; ** $p < 0.005$ *** $p < 0.001$.

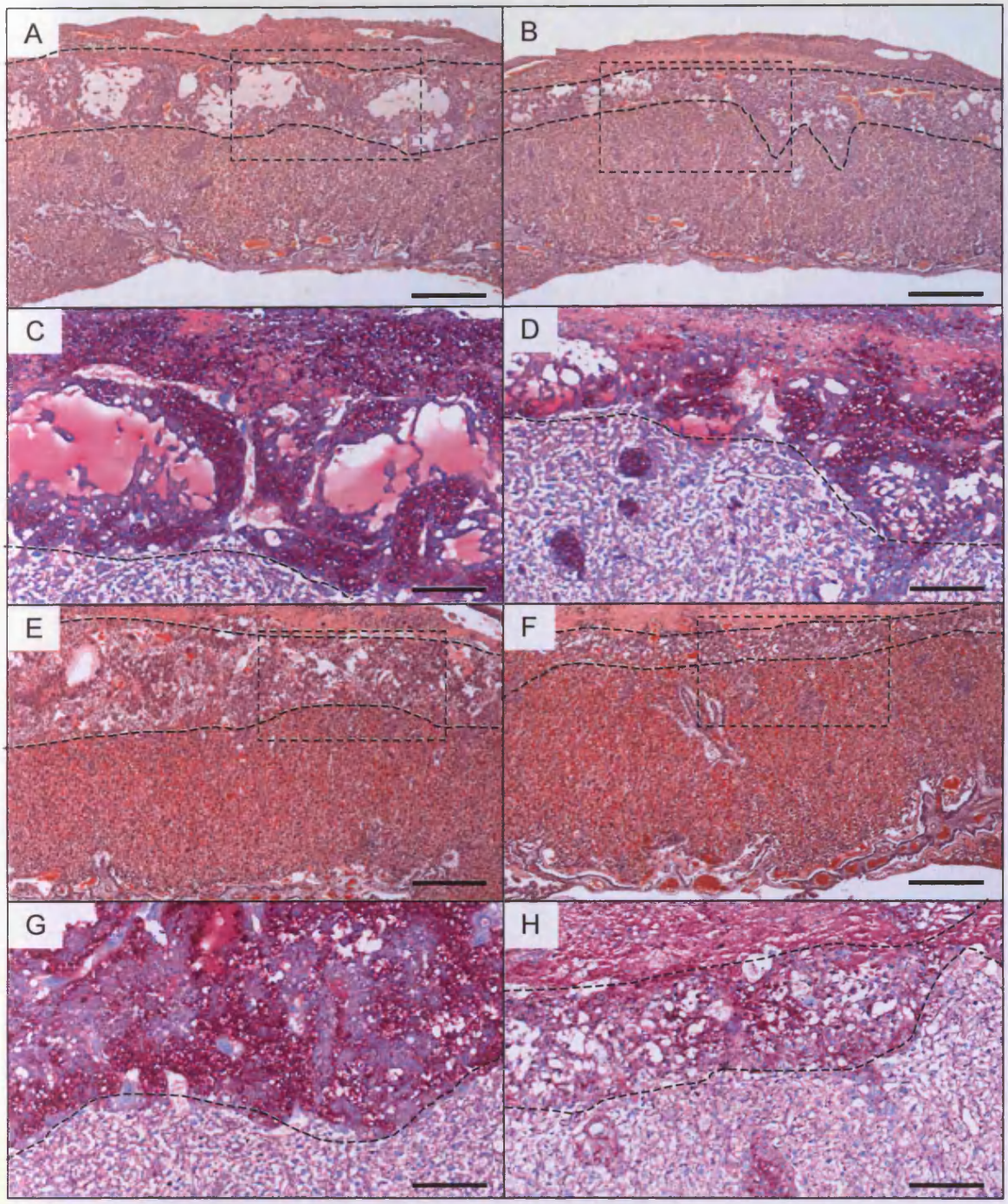


Figure 4.10: Glycogen cells in placentae of line 10-10 on the 129/Sv x C57BL/6 background

General placental morphology was examined by H&E staining, with relative glycogen cell number assessed by PAS staining. H&E (A, B, E, F) and PAS (C, D, G, H) staining at E14.5 (upper four) and E18.5 (lower four) of wild type (left column) and transgenic (right column) placentae. Dashed boxes indicate region of section stained for glycogen cells and shown at higher magnification below. Broken lines indicate boundaries of placental layers; labyrinth at bottom. Scale bar = 500 μm (H&E) and 200 μm (PAS).

4.2.3 Effect of genetic background on wild type placenta

Wild type placentae on a pure 129/Sv background were overtly smaller compared to wild type placentae on the 129/Sv x C57BL/6 background at both E14.5 and E18.5 (Fig 4.11). Normalised wild type placental weights from 129/Sv x C57BL/6 litters of lines 10-15 and 10-10 were combined and compared to wild type placental weights from line 10-15 on a pure 129/Sv background. By weight, wild type 129/Sv x C57BL/6 placentae were 38.1% heavier than pure 129/Sv wild type placentae at E14.5 (78.7 mg ± 12.5 versus 108.6 mg ± 9.2; wild type placentae from 19 litters; n = 73; $p = 2.10 \times 10^{-18}$). A similar difference in weight persisted at E18.5, with 129/Sv x C57BL/6 placentae 45.1% heavier than pure 129/Sv wild type (79.6 mg ± 6.9 versus 115.4 mg ± 7.2; wild type placentae from 22 litters; n = 82; $p = 7.22 \times 10^{-37}$).

The increased weight of placentae on the 129/Sv x C57BL/6 background appears to be due entirely to the presence of a larger spongiotrophoblast area, which is 2.34 times larger in placentae of the 129/Sv x C57BL/6 background compared to those on the pure 129/Sv background (1.36 mm^2 ± 0.40 versus 3.17 mm^2 ± 0.61; wild type placentae from 11 litters; n = 37; $p = 3.29 \times 10^{-6}$). In contrast, there was no significant difference in the area of the labyrinth region (3.66 mm^2 ± 0.36 versus 3.85 mm^2 ± 0.79; $p = 0.104$). The difference in the combined area of spongiotrophoblast and labyrinth regions was comparable to the weight difference between the two genetic backgrounds, with a 37% increased area in placentae of the 129/Sv x C57BL/6 background (5.10 mm^2 ± 0.41 versus 7.00 mm^2 ± 1.04; $p = 7.17 \times 10^{-5}$) (Fig 4.12).

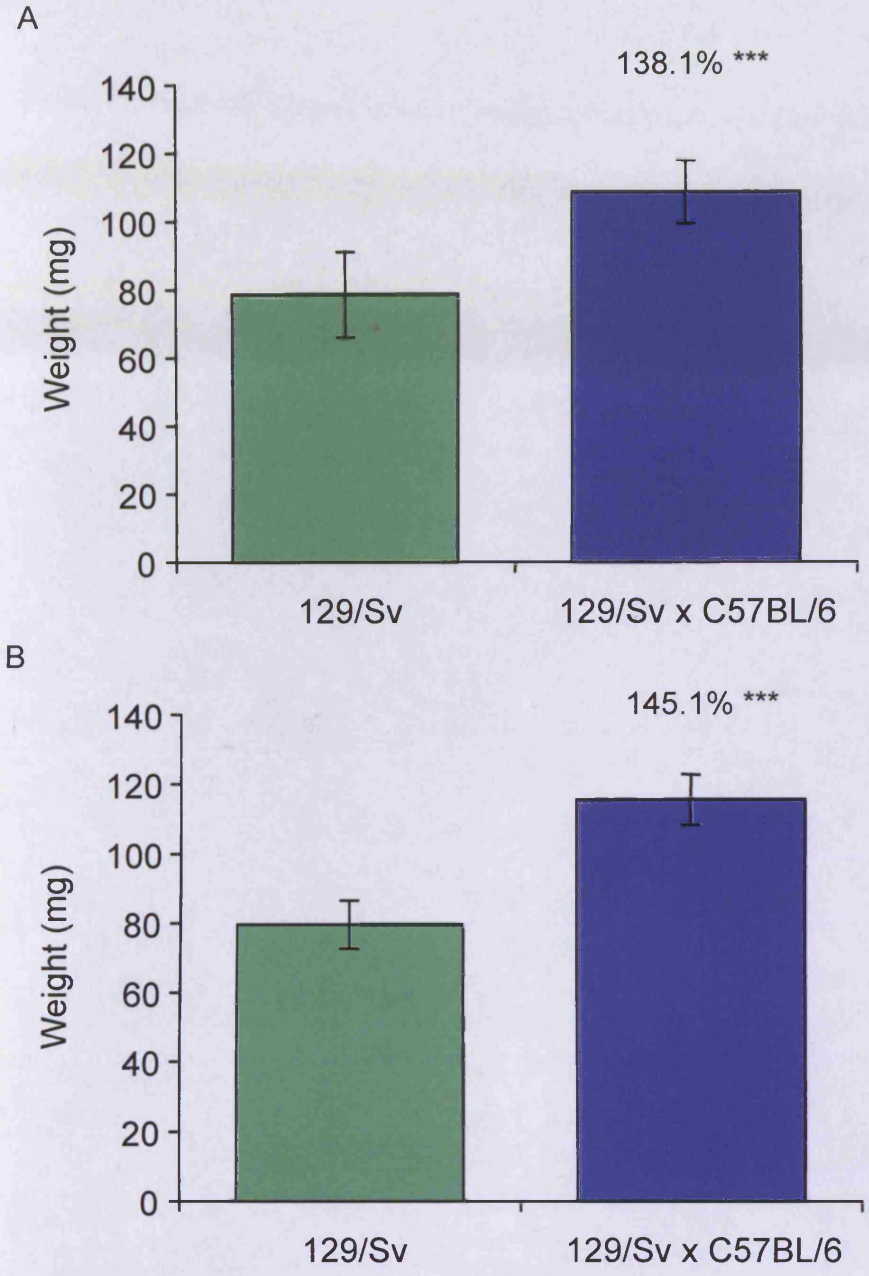


Figure 4.11: Comparison of wild type placental weights at E14.5 and E18.5 between pure 129/Sv and 129/Sv x C57BL/6 backgrounds

Wild type placental weight was compared between the two genetic backgrounds used. Mean normalised weights of wild type placentae are shown on the 129/Sv and 129/Sv x C57BL/6 genetic backgrounds at A) E14.5 and B) E18.5. Percentage values indicate the ratio of mean placental weight on the 129/Sv x C57BL/6 background to that on the 129/Sv background. Error bars show standard deviation and asterisks denote significance level according to the Student's *t*-test: *** $p < 0.001$.

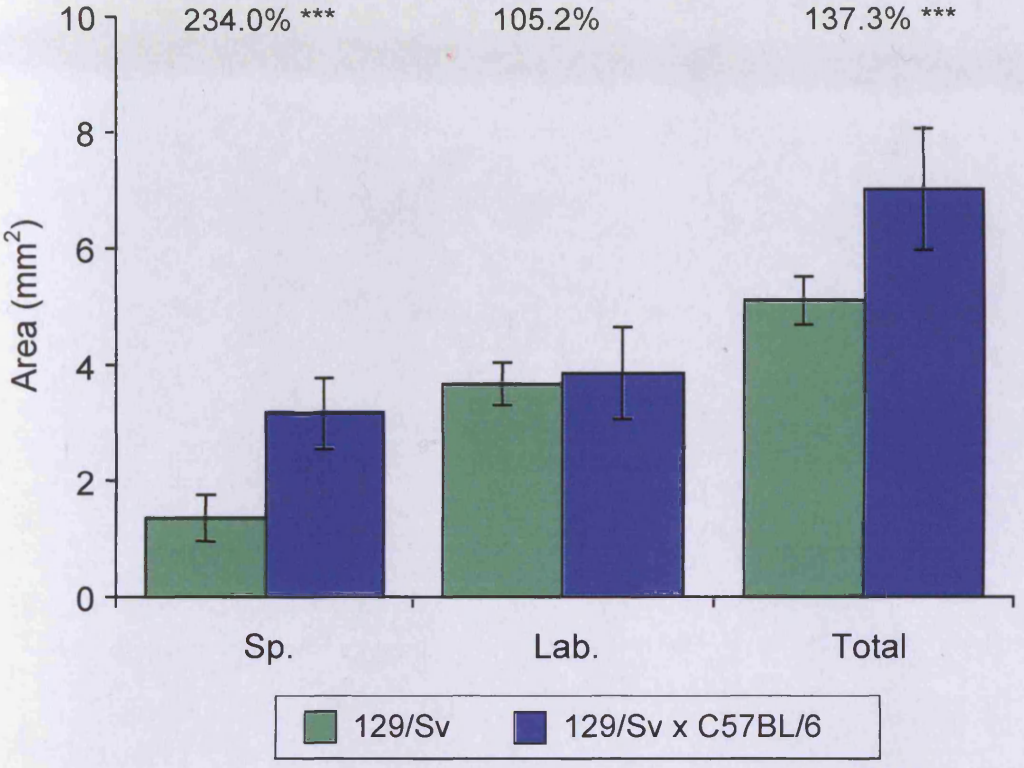


Figure 4.12: Comparison of midline placental areas at E14.5 on a pure 129/Sv and 129/Sv x C57BL/6 genetic background

To determine whether placentae on a 129/Sv x C57BL/6 background were proportionately larger than those on a pure 129/Sv background, spongiotrophoblast and labyrinth areas were compared. Graph shows mean midline areas of spongiotrophoblast (Sp.), labyrinth (Lab.) and combined spongiotrophoblast and labyrinth regions (Total), with error bars representing standard deviation. Percentage values indicate the ratio of midline area on a 129/Sv x C57BL/6 background to the area on a pure 129/Sv background. Asterisks denote significance level according to the Mann-Whitney test: *** $p < 0.001$.

4.2.4 Characterising line 5D3 on the 129/Sv x C57BL/6 background

The effect of over-expressing *Phlda2* and *Slc22a18* in the mouse placenta was characterised in two independent transgenic lines, one possessing three additional copies of each gene, and the other with an undetermined transgene copy number. The key placental phenotypes of both transgenic lines were comparable, indicating that the transgene integration site was not a significant contributory factor to the phenotype. Both lines 10-15 and 10-10 possess more than one copy of the BAC transgene. Loss of imprinting of the IC2 domain would be equivalent to the presence of two active copies of the genes investigated in this study. We next examined whether a lower dosage of *Phlda2* and *Slc22a18* showed a similarly severe placental phenotype by making use of a single copy transgenic line (5D3) and a two-copy transgenic line (5A4). The transgene present in these transgenic lines contains an intact *Cdkn1c* gene, but since the *Cdkn1c* placenta-specific enhancers are not on the BAC (John *et al.* 2001), lines 5D3 and 5A4 can be compared with lines 10-15 and 10-10, as only *Phlda2* and *Slc22a18* are over-expressed in the placenta of all three lines.

Transgene copy number was previously determined by Southern blotting, with gene expression further assessed by qPCR. At E12.5, expression of *Phlda2* in transgenic placentae on the 129/Sv x C57BL/6 background was 4.9-fold (± 1.3) compared to wild type with *Slc22a18* expression elevated by 4.6-fold (± 1.8) (Fig 4.13A). To examine if the inconsistency between observed gene expression and the anticipated expression level predicted by the transgene copy number was due to genetic background differences, expression was also assessed from generation 7 C57BL/6 placentae by qPCR. Expression of *Phlda2* and *Slc22a18* correlated more closely with the predicted expression level, with *Phlda2* upregulated by 2.3-fold (± 0.9) compared to wild type and *Slc22a18* expression elevated by 1.6-fold (± 0.8) (Fig 4.13B). However, as exemplified by the large standard deviations, expression levels remained variable between the individual placentae that were compared.

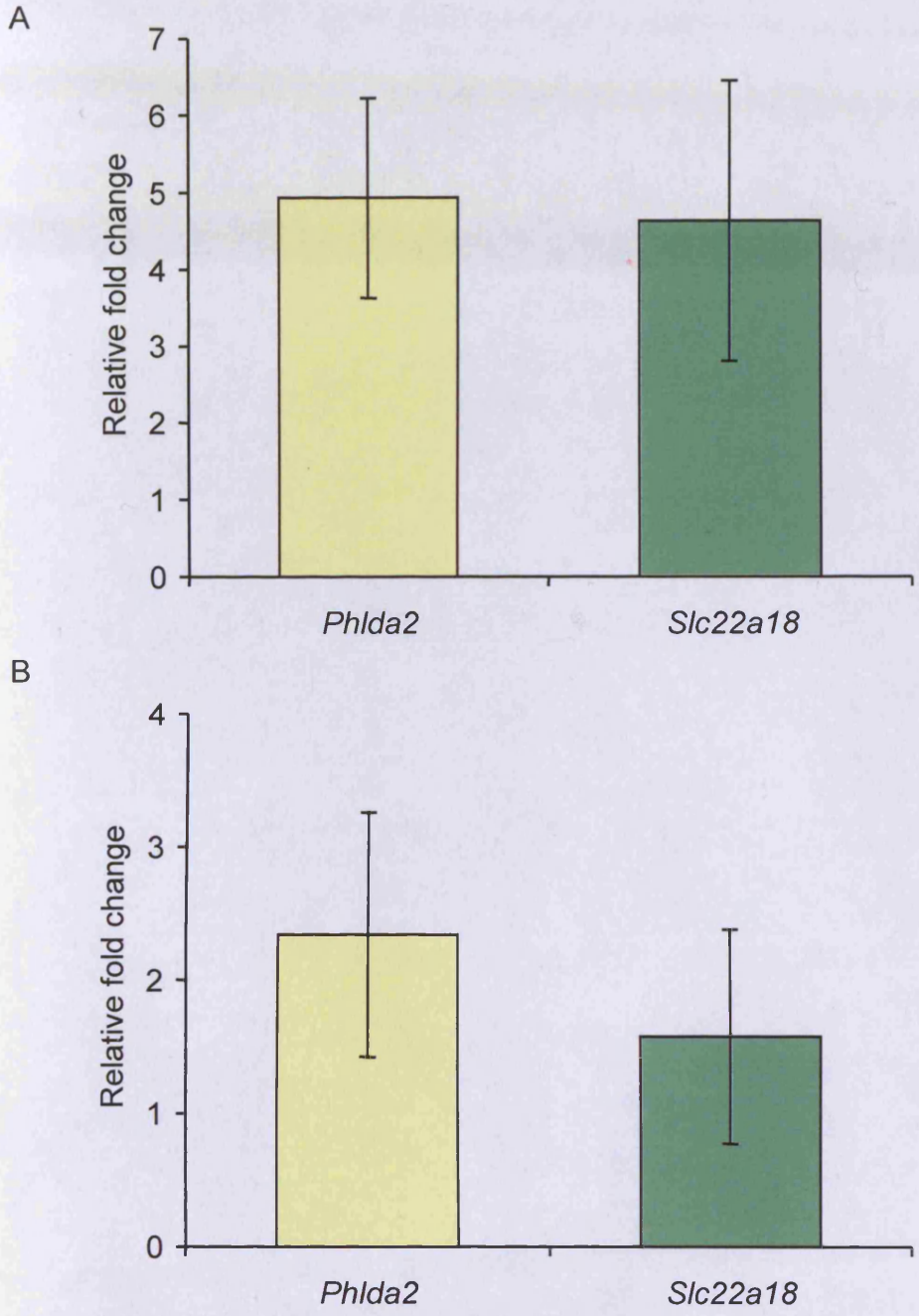


Figure 4.13: Transgene expression at E12.5 in placentae of line 5D3 on two genetic backgrounds

Expression from the transgene was first examined on the 129/Sv x C57BL/6 background, and subsequently from a G7 C57BL/6 mating. Fold change between wild type and transgenic placentae of line 5D3 on A) 129/Sv x C57BL/6 genetic background and B) G7 C57BL/6 genetic background. Error bars show standard deviation.

The placental phenotype was examined in line 5D3 on the 129/Sv x C57BL/6 background. At E13.5 transgenic placentae were approximately 27% lighter than wild type (87.1 mg ± 9.8 versus 63.9 mg ± 13.1; 5 litters; n = 40; p = 1.98 x 10^{-6}), a phenotype which persisted to a similar degree at E14.5 (106.5 mg ± 8.8 versus 78.3 mg ± 8.4; 3 litters; n = 22; p = 4.40 x 10^{-7}) and E18.5 (110.1 mg ± 12.3 versus 84.8 mg ± 10.8; 7 litters; n = 59; p = 6.51 x 10^{-8}) (Fig 4.14A). A more severe placental weight deficit was observed in transgenic placentae of line 5A4, which possess two copies of the unmodified BAC transgene. At E13.5 transgenic placentae were approximately 40% lighter than wild type (90.2 mg ± 12.9 versus 54.1 mg ± 12.5; 7 litters; n = 50; p = 1.08 x 10^{-6}), with a similar difference at E14.5 (107.0 mg ± 3.9 versus 66.2 mg ± 5.5; 4 litters; n = 25; p = 3.56 x 10^{-16}), and a ~30% weight deficit at E18.5 (115.5 mg ± 10.7 versus 81.5 mg ± 12.7; 7 litters; n = 54; p = 2.04 x 10^{-14}) (Fig 4.14B).

At E14.5, spongiotrophoblast area was significantly reduced in transgenic placentae, accounting for only 40% of the area in wild type placentae (3.18 mm^2 ± 0.68 versus 1.28 mm^2 ± 0.27; 2 litters; n = 17; p = 7.24 x 10^{-3}) (Fig 4.15B). No significant effect on labyrinth area was observed (4.38 mm^2 ± 0.65 versus 4.04 mm^2 ± 0.44; 2 litters; n = 17; p = 0.831), with the combined area of the two regions reduced by approximately 30% (7.64 mm^2 ± 0.97 versus 5.32 mm^2 ± 0.52; 2 litters; n = 13; p = 7.08 x 10^{-3}). Furthermore, the mean spongiotrophoblast area of transgenic placentae from line 5D3 was significantly less than that of transgenic placentae of lines 10-15 and 10-10 on the 129/Sv x C57BL/6 background (see Table 4.1). Glycogen staining was reduced although correctly localised as seen in transgenic placentae from both lines 10-15 and 10-10 (Fig 4.17).

In summary, transgenic mice that possess two active copies of *Phlda2* and *Slc22a18* exhibit a placental phenotype that is similar to that of lines 10-15 and 10-10. Placental weight was reduced to a comparable degree, although the loss of spongiotrophoblast area in line 5D3 was more severe than observed in lines 10-15 and 10-10. Placental weight of transgenic placentae from line 5A4 was more severely reduced that observed in lines 10-15, 10-10 and 5D3.

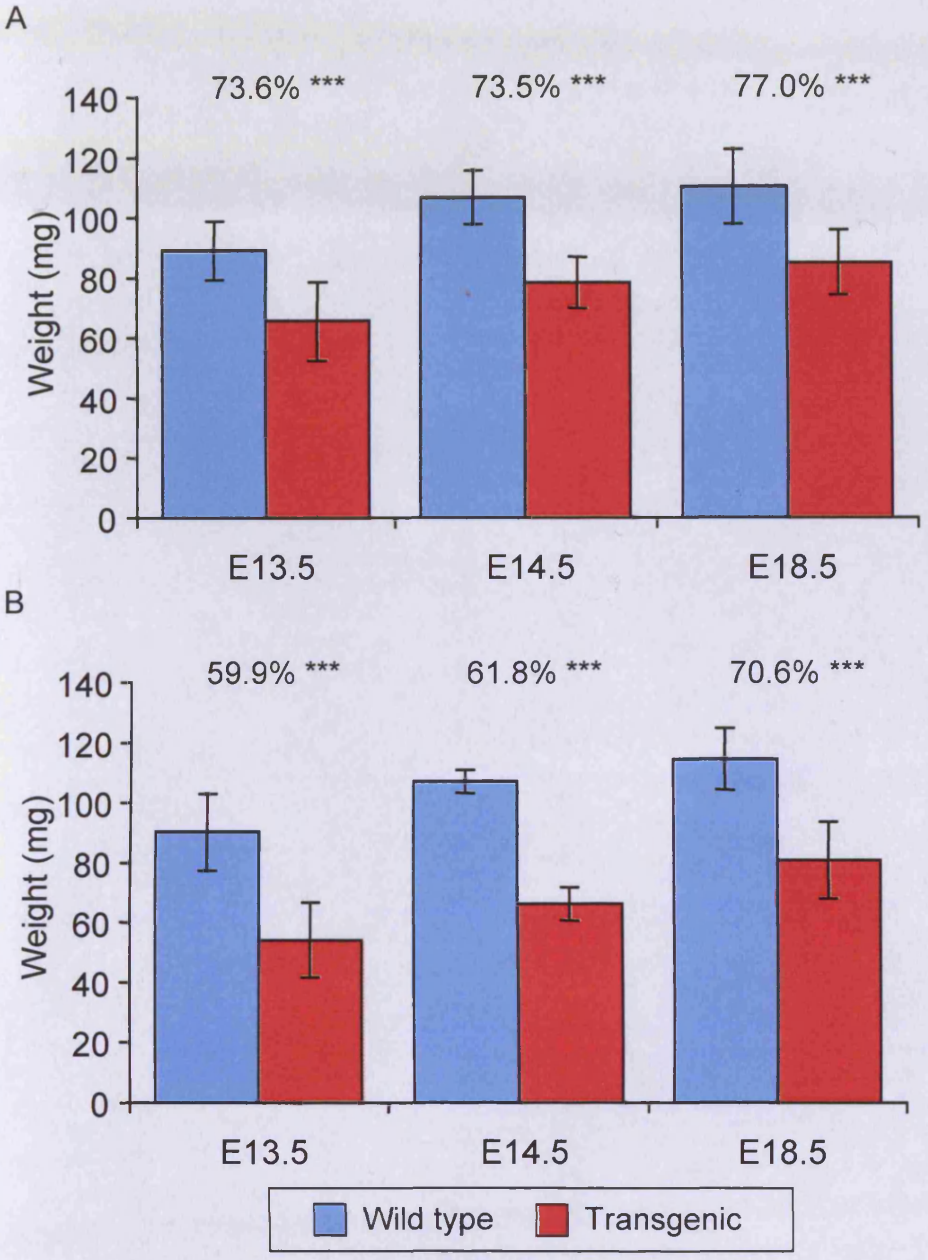


Figure 4.14: Placental weights from lines 5D3 and 5A4 on the 129/Sv x C57BL/6 background

Placental weights were compared between lines 5D3 and 5A4 at E13.5, E14.5 and E18.5. Graphs show mean normalised placental weight of wild type and transgenic placentae of A) line 5D3 on the 129/Sv x C57BL/6 background at E13.5, E14.5 and E18.5 and B) line 5A4 at E13.5, E14.5 and E18.5. Percentage values indicate the ratio of transgenic weight to wild type. Error bars represent standard deviation. Statistical significance as determined by *t*-test is denoted by asterisks *** $p < 0.001$.

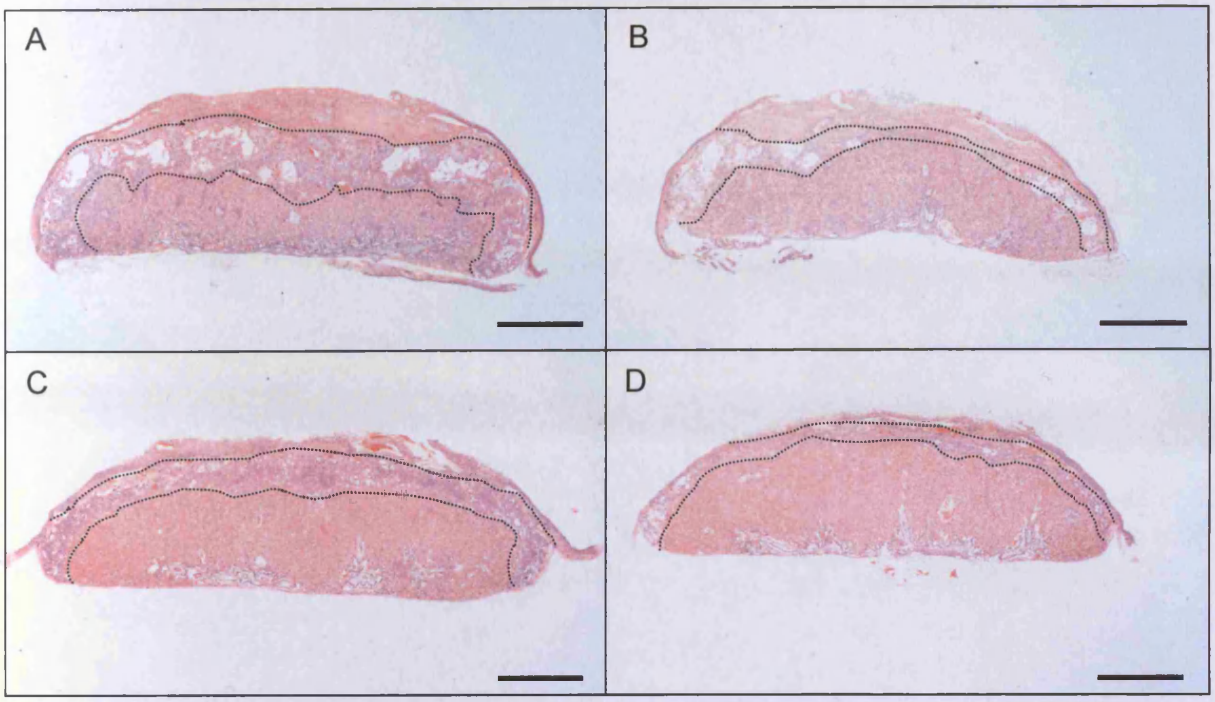


Figure 4.15: Loss of spongiotrophoblast in line 5D3 on the 129/Sv x C57BL/6 background

Placental morphology was examined by H&E staining of wild type (A and C) and transgenic (B and D) placentae at E14.5 (A and B) and E18.5 (C and D) from line 5D3 on the 129/Sv x C57BL/6 background. Dashed lines indicate boundary of spongiotrophoblast. Scale bar = 1 mm.

	Area (mm^2)	% of WT area	*P*
5D3	1.28 ± 0.27	40%	-
10-15	1.81 ± 0.42	57%	0.00387
10-10	1.74 ± 0.49	55%	0.0190
Combined 10-15 and 10-10	1.77 ± 0.45	56%	0.00421

Table 4.1: Comparison of mean spongiotrophoblast area of transgenic placentae from lines 10-15, 10-10 and 5D3 on the 129/Sv x C57BL/6 background

The table shows the mean spongiotrophoblast area of transgenic placentae from lines 5D3, 10-15, 10-10 and the combined data for lines 10-15 and 10-10. The midline spongiotrophoblast area of transgenic placentae from line 5D3 was significantly smaller than that of lines 10-15 and 10-10. The *p* value represents a comparison between the spongiotrophoblast area of transgenic placentae of line 5D3 and the area of the designated line on the 129/Sv x C57BL/6 background.

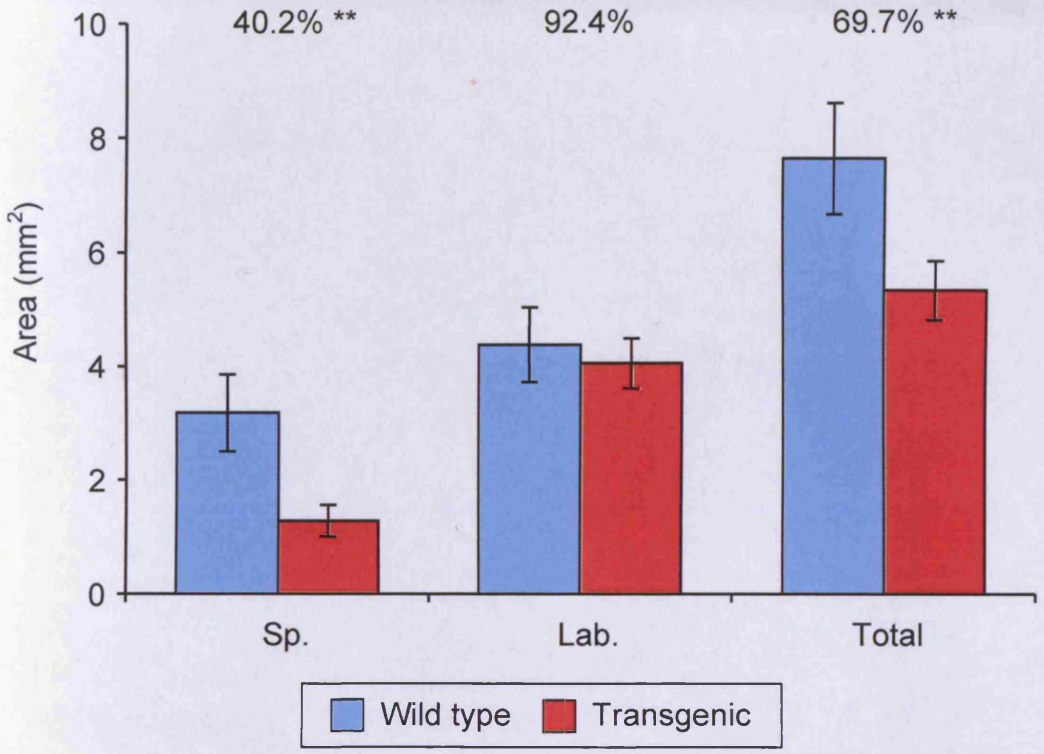


Figure 4.16: Midline placental areas in line 5D3 on the 129/Sv x C57BL/6 background

Spongiotrophoblast (Sp.), labyrinth (Lab.) and combined (Total) areas were measured from midline placental sections of line 5D3 at E14.5. Percentage values indicate the ratio of transgenic area to wild type. Error bars show standard deviation. Statistical significance as determined by Mann-Whitney test is denoted by asterisks; ** $p < 0.005$.

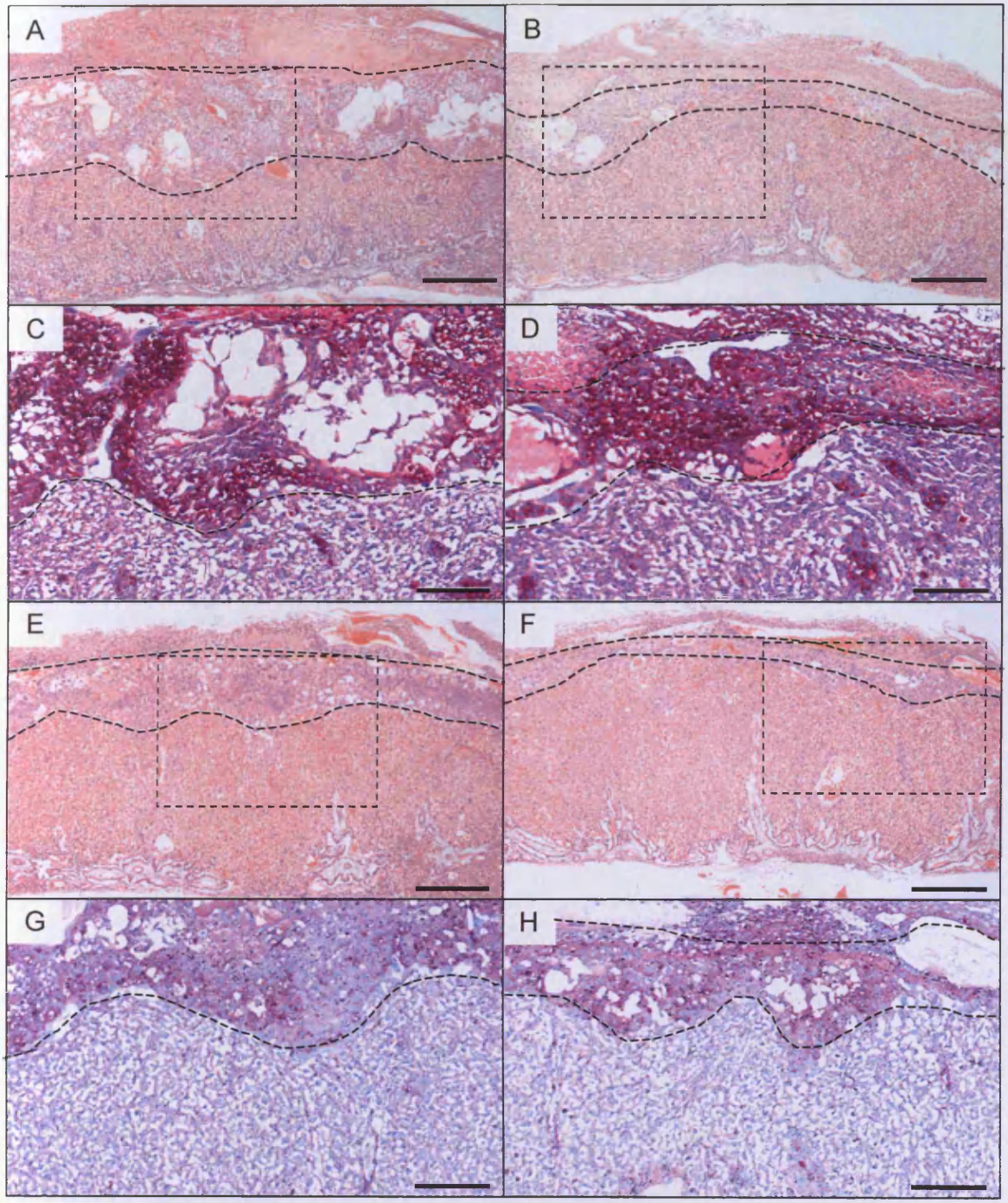


Figure 4.17: Glycogen cells in placentae of line 5D3 on the 129/Sv x C57BL/6 background

H&E (A, B, E, F) and PAS (C, D, G, H) staining at E14.5 (upper four) and E18.5 (lower four) of wild type (left column) and transgenic (right column) placentae. Dashed box indicates region of section stained for glycogen cells and shown at higher magnification below. Broken lines indicate boundaries of placental layers; labyrinth at bottom. Scale bar = 500 μm (H&E) and 200 μm (PAS).

4.2.5 Restoration of *Phlda2* gene dosage to normal to assess the role of *Slc22a18* in the placental phenotype

All transgenic lines utilised throughout this investigation over-express both *Phlda2* and *Slc22a18* in the placenta. Lines 10-15 and 10-10 contain modified BAC transgenes, whereby *Cdkn1c* is inactivated by the presence of a *β-galactosidase* reporter construct. Enhancers required for placental *Cdkn1c* expression are not located on the transgene, thus *Cdkn1c* is not over-expressed in placentae of lines 5D3 or 5A4 (John *et al.* 2001).

A consequence of over-expressing two genes is that the resulting phenotype cannot be directly attributed to either gene. Previously published observations however, indicate that the placental deficit and reduced spongiotrophoblast observed in this model are primarily due to over-expression of *Phlda2*. Firstly, the small placenta and reduced spongiotrophoblast characteristic of transgenic placentae is the reciprocal to the phenotype of *Phlda2* null placentae, which exhibit placentomegaly and an increased spongiotrophoblast region (Frank *et al.* 2002). Furthermore, in mice that lack paternal *Kvdmr1*, and thus over express *Phlda2* and *Slc22a18*, the placenta is reduced in size with a small spongiotrophoblast. Restoration of *Phlda2* gene dosage to normal by paternal inheritance of the *Kvdmr1* null allele with maternal inheritance of the *Phlda2* null allele rescued both placental weight and spongiotrophoblast phenotypes (Salas *et al.* 2004).

A similar approach was undertaken in an attempt to dissect out the contribution of *Phlda2* and *Slc22a18* to the placental phenotype. Mating a stud male from the single copy transgenic line (5D3) with a female *Phlda2* null mouse, generated progeny that possess both the transgene and the *Phlda2* null allele. These progeny thus have two active *Slc22a18* alleles but only one active *Phlda2* allele, which is located on the transgene. By investigating the effect of restoring *Phlda2* gene dosage to normal, it was envisaged that the contribution of over-expression of this gene to the phenotype would be revealed. Utilising both heterozygous male and female mice enabled four genotypes to be generated in each litter, with an expected Mendelian ratio of 1:1:1:1 (Fig 4.1). In doing so, a direct comparison of wild type, transgenic and "rescue" placentae was possible in placentae from the same litter.

		Maternal	
		Wild type	***Phlda2*$^{-/+}$**
Paternal	**Wild type**	Wild type	*Phlda2*$^{-/+}$
	Transgene	Transgenic	*Phlda2*$^{-/+}$ with transgene

Punnett Square showing resulting genotypes from a mating between a hemizygous 5D3 transgenic male and a heterozygous *Phlda2* null female.

Table 4.2: Punnett square showing potential genotypes generated in double transgenic matings

The four potential genotypes generated in such a breeding program differ according to the number of active alleles of *Phlda2*, *Slc22a18* and *Cdkn1c* (Table 4.2). Wild type conceptuses possess a single active allele for each gene, whereas transgenic conceptuses possess two active alleles for *Phlda2* and *Slc22a18*, and a single active *Cdkn1c* allele. *Phlda2* null conceptuses possess a single active allele for *Slc22a18* and *Cdkn1c*, but no active *Phlda2* allele. The combined inheritance of the transgene and *Phlda2* null allele results in two copies of *Slc22a18*, while *Phlda2* gene dosage is maintained at normal level of one active allele.

	Phlda2	***Slc22a18***	***Cdkn1c***
Wild type	1	1	1
Transgenic	2	2	1†
***Phlda2*$^{-/+}$**	0	1	1
***Phlda2*$^{-/+}$ with transgene**	1	2	1†

Table 4.3: Number of active alleles according to genotype in the placentae of conceptuses from double transgenic matings

† *Cdkn1c* is represented by the presence of two alleles in these progeny, although they are only actively expressed in the embryo, as *Cdkn1c* is not expressed from the transgene in the placenta.

Phlda2 expression was quantified by qPCR at E12.5 to determine whether expression levels were restored to wild type levels in the double transgenic placentae. Optimally, a direct comparison of *Phlda2* expression would be made between double transgenic and wild type placentae. However, this was not possible, as none of the four litters collected at E12.5 contained two wild type and two double transgenic placentae. Instead, expression of *Phlda2* and *Slc22a18* was compared between double transgenic and 5D3 transgenic placentae. Assuming that appropriate copy number-dependent *Phlda2* expression exists from the transgene, such a comparison would be expected to yield a 2-fold reduction in *Phlda2* levels with *Slc22a18* expression remaining comparable between the two genotypes. Consistent with this prediction, *Phlda2* expression was reduced by 2.24-fold (± 0.13) in double transgenics, whereas *Slc22a18* remained unaffected (0.96-fold ± 0.08) (Fig 4.18). Thus, *Phlda2* expression was reduced in double transgenic placentae compared to 5D3 transgenic placentae.

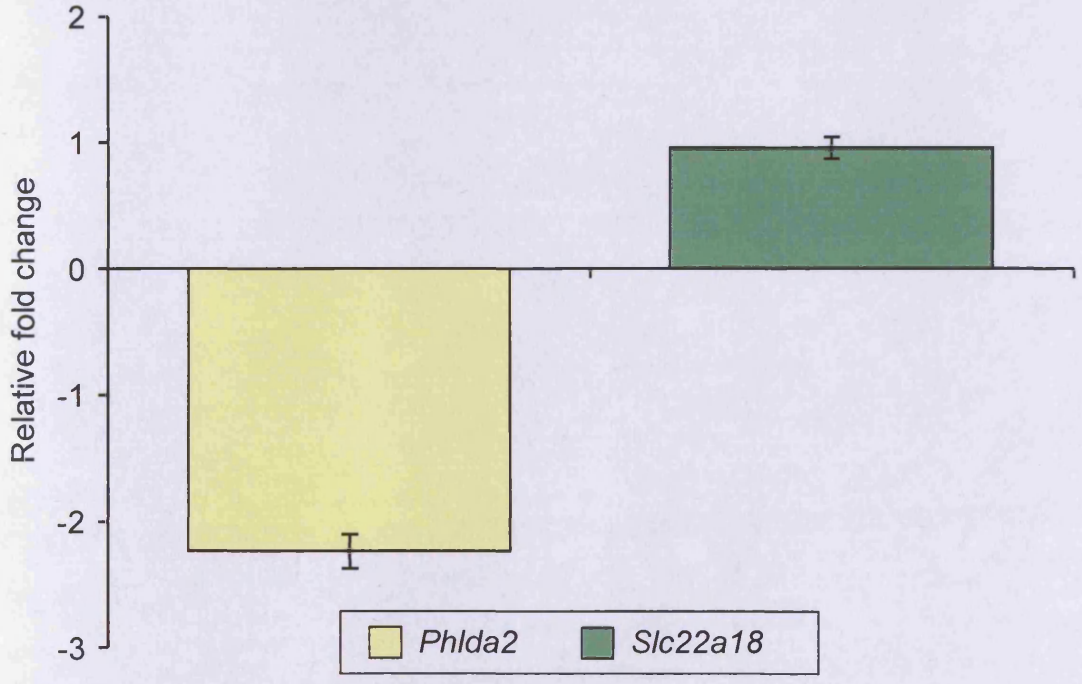


Figure 4.18: Relative expression levels of *Phlda2* and *Slc22a18*

Placental expression of *Phlda2* and *Slc22a18* in double transgenic placenta compared with 5D3 transgenic placentae. Graph shows mean fold change with error bars representing standard deviation.

Expression of *Phlda2* was also examined at E13.5 by *in situ* hybridisation (Fig 4.17A-D). Comparison of *Phlda2* probe hybridisation between wild type, transgenic and double transgenic placentae indicated that *Phlda2* expression was most similar between wild type (Fig 4.19A) and double transgenic placentae (Fig 4.19C). Although not a quantitative measure, comparison of signal intensity between wild type and single BAC transgenic placentae (Fig 4.19B) indicated that *Phlda2* expression was greater than two-fold wild-type levels in this individual sample. This is consistent with qPCR analysis of expression from placentae of line 5D3 on the 129/Sv x C57BL/6 background, which suggests that *Phlda2* expression can vary. Thus, although *Phlda2* expression was reduced in double transgenic placentae compared to 5D3 transgenic placentae, expression levels appear to remain elevated above wild type levels. As anticipated, no signal was observed in *Phlda2* null placentae (Fig 4.17D).

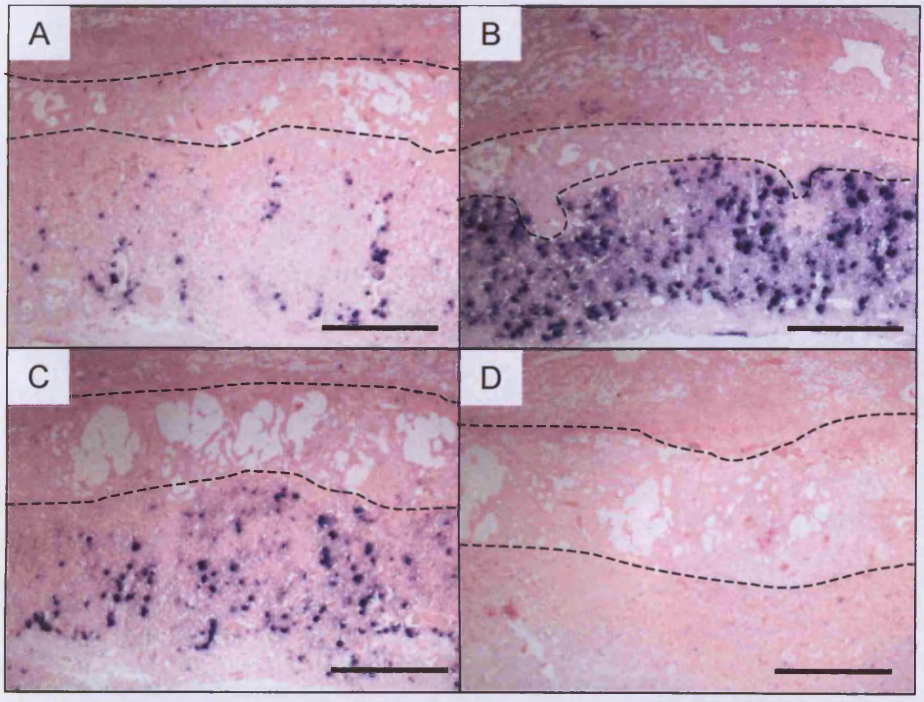


Figure 4.19: *Phlda2* expression at E13.5 in each of the four genotypes generated by double transgenic matings

Expression of *Phlda2* was examined by *in situ* hybridisation at E13.5 in placentae from double transgenic matings. A) Wild type, B) 5D3 transgenic, C) 5D3 transgenic and *Phlda2* null, D) *Phlda2* null. Broken lines indicate boundaries of placental layers: labyrinth at bottom. Scale bar = 500 μm.

Placentae and embryos were dissected at E14.5 and wet weights recorded as described previously. Fourteen litters were obtained at E14.5, comprising 24 wild type, 24 transgenic, 34 *Phlda2* null and 18 double transgenic conceptuses. Due to the four potential genotypes arising in each litter, only nine of the 14 litters contained at least two wild-type conceptuses. All data collected were first analysed as raw data (Fig 4.20 A). Consistent with results reported in Section 4.2.3, transgenic placentae were 20% lighter than wild type at E14.5 (106.0 mg ± 18.71 versus 84.1 mg ± 13.88; 14 litters; n = 48; $p = 3.22 \times 10^{-5}$). Furthermore, and consistent with previously published results, *Phlda2* null placentae were significantly heavier than wild type (106.0 mg ± 18.71 versus 132.1 mg ± 24.21; 14 litters; n = 58; $p = 4.36 \times 10^{-5}$). The double transgenic placentae were 8% lighter than wild type placentae, but this result was not statistically significant at the 5% significance level (106.0 mg ± 18.71 versus 97.7 mg ± 14.86; 14 litters; n = 42; $p = 0.128$). In contrast, the mean weight of double transgenic placentae was significantly greater than that of 5D3 transgenic placentae (84.1 mg ± 13.88 versus 97.7 mg ± 14.86; 14 litters; n = 42; $p = 4.1 \times 10^{-3}$).

The weights obtained from the nine litters with at least two wild type conceptuses were normalised and analysed separately. A similar pattern of results was observed; transgenic placentae were 15.2% lighter than wild type (104.0 mg ± 16.5 versus 83.8 mg ± 21.4; 9 litters; n = 33; $p = 0.0167$), and *Phlda2* null placentae were significantly heavier than wild type (104.0 mg ± 16.5 versus 118.8 mg ± 25.9; 9 litters; n = 43; $p = 5.37 \times 10^{-3}$). Double transgenic placentae appeared to be 10.5% lighter than wild type, although this failed to achieve statistical significance (104.0 mg ± 16.5 versus 93.1 mg ± 12.2; 9 litters; n = 21; $p = 0.0741$). In contrast to analysis of raw data however, there was no significant difference between the mean weights of 5D3 transgenic or double transgenic placentae (87.2 mg ± 21.4 versus 93.1 mg ± 12.2; 9 litters; n = 22; $p = 0.446$).

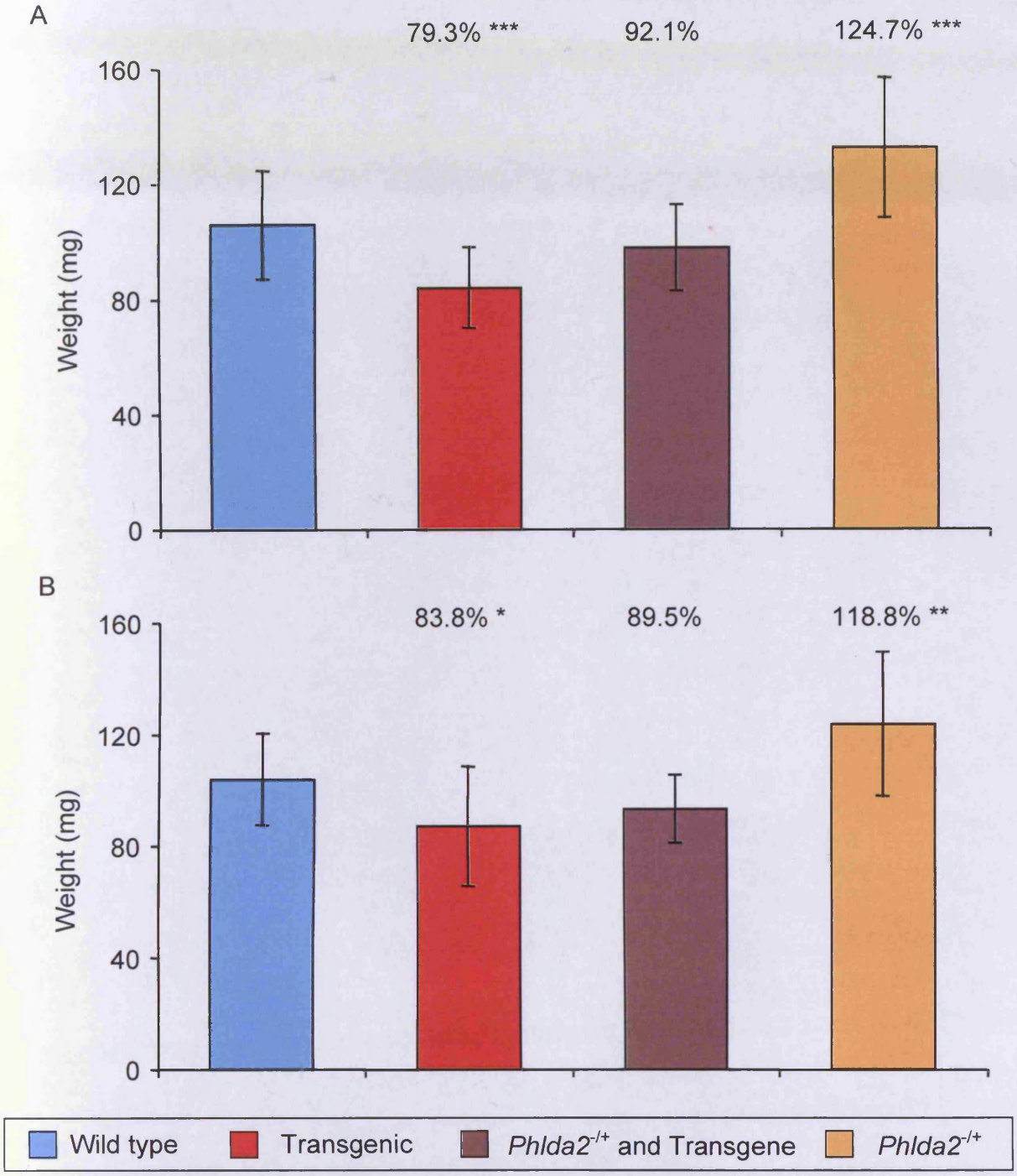


Figure 4.20: Placental weights from double transgenic matings at E14.5

Mean (A) raw and (B) normalised placental weights from each genotype at E14.5 from a cross between 5D3 and *Phlda2*$^{-/+}$. Graphs show mean weight, with error bars denoting standard deviation. Percentage values indicate the weight ratio relative to wild type. Significance level calculated by the *t*-test is indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Sections from placentae representing each of the four genotypes were hybridised with the *Tpbpa* probe at E14.5 and E18.5 to examine the relative spongiotrophoblast area in each genotype (Fig 4.21). The mislocalisation of *Tpbpa*-positive cells in the labyrinth, which was a prominent feature of transgenic placentae at both E14.5 and E18.5, was comparably less severe in transgenic placentae examined from double transgenic matings. Spongiotrophoblast of rescue placentae appeared larger than that of transgenic placentae. To determine the extent of this apparent return to the wild type state, spongiotrophoblast area was measured from midline sections of placentae from a number of litters (Fig 4.22).

As anticipated, spongiotrophoblast area was significantly reduced in transgenic placentae, representing approximately 44% the spongiotrophoblast area observed in wild type (3.52 mm^2 ± 0.57 versus 1.58 mm^2 ± 0.49; 5 litters; n = 19; $p = 2.80 \times 10^{-4}$). Spongiotrophoblast area in rescue placentae was approximately 70% that of wild type (3.52 mm^2 ± 0.57 versus 2.40 mm^2 ± 0.20; 5 litters; n = 20; $p = 1.09 \times 10^{-3}$), although this represented a significant ~50% increase over the transgenic placentae (1.58 mm^2 ± 0.49 versus 2.40 mm^2 ± 0.20; 5 litters; n = 21; $p = 2.87 \times 10^{-4}$). In agreement with previously published results, the spongiotrophoblast area of *Phlda2* null placentae was approximately 40% larger (3.52 mm^2 ± 0.57 versus 4.77 mm^2 ± 1.18; 5 litters; n = 18; $p = 5.02 \times 10^{-3}$). The extent of glycogen staining at E14.5 and E18.5 appeared to correlate with spongiotrophoblast area (Figs 4.23 and 4.24). Increased glycogen staining was observed in double transgenic placentae in comparison to 5D3 transgenic placentae, although visibly less than wild type placentae at the same stage. As expected, glycogen staining was overtly stronger in *Phlda2* null placentae.

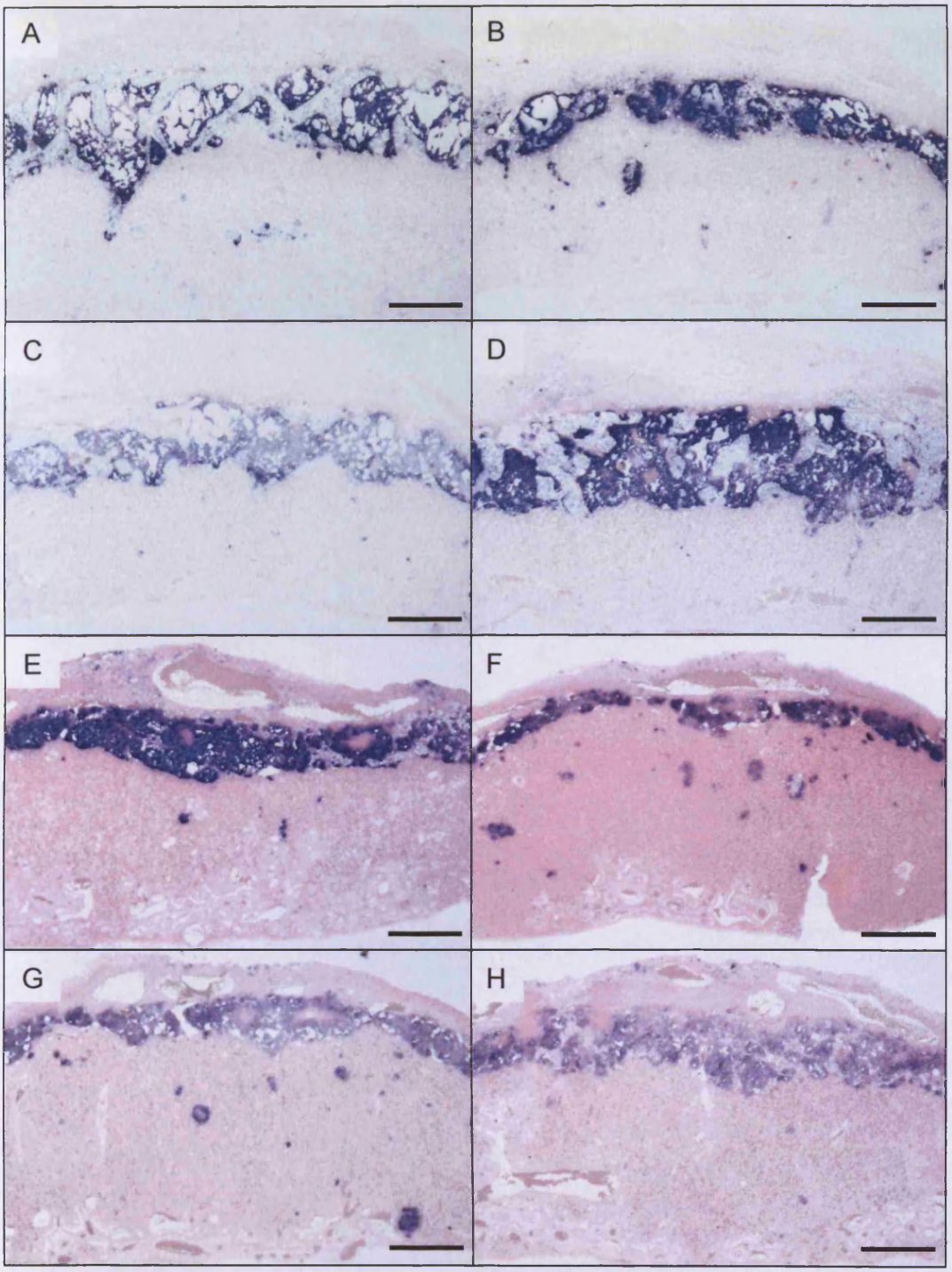


Figure 4.21: Assessment of spongiotrophoblast in placenta from double transgenic matings

Spongiotrophoblast in placentae from double transgenic matings. E14.5 (A-D) and E18.5 (E-H). A and E) Wild type, B and F) 5D3 transgenic, C and G) 5D3 transgenic and *Phlda2* null, D and H) *Phlda2* null. Scale bar = 500 μm.

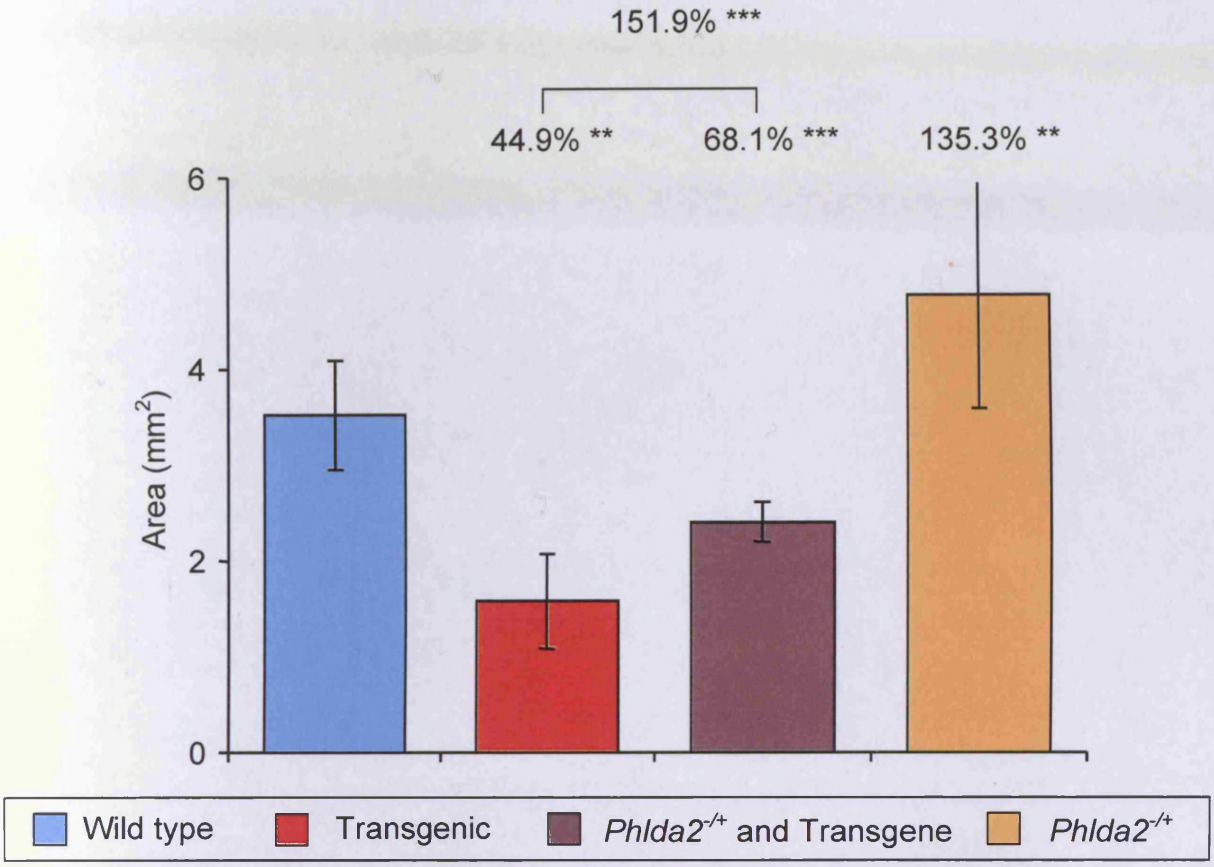


Figure 4.22: Spongiotrophoblast area from double transgenic matings

Spongiotrophoblast area was measured from midline placental sections at E14.5. Error bars show standard deviation. Percentage values indicate the weight ratio relative to wild type. Statistical significance as determined by Mann-Whitney test is denoted by asterisks; ** $p < 0.005$ *** $p < 0.001$.

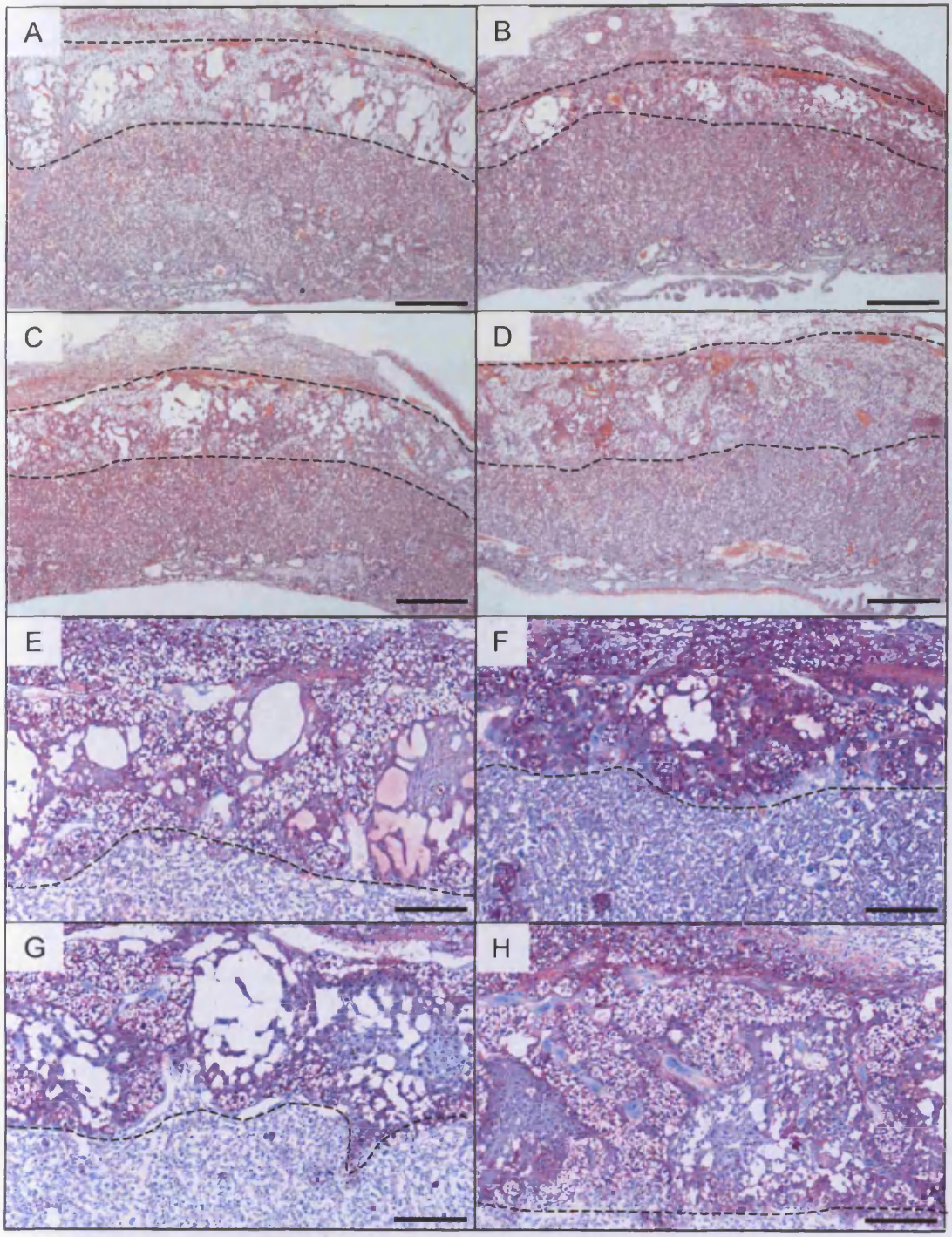


Figure 4.23: Glycogen staining at E14.5 in double transgenic matings

Placental morphology was examined from H&E stained sections at E14.5 (upper panels) and glycogen cells detected by PAS staining (lower panels). A and E) Wild type, B and F) 5D3 transgenic, C and G) 5D3 transgenic and *Phlda2* null, D and H) *Phlda2* null. Broken lines indicate boundaries between placental layers: labyrinth at bottom. Scale bar = 500 μm for A-D and 200 μm for E-H.

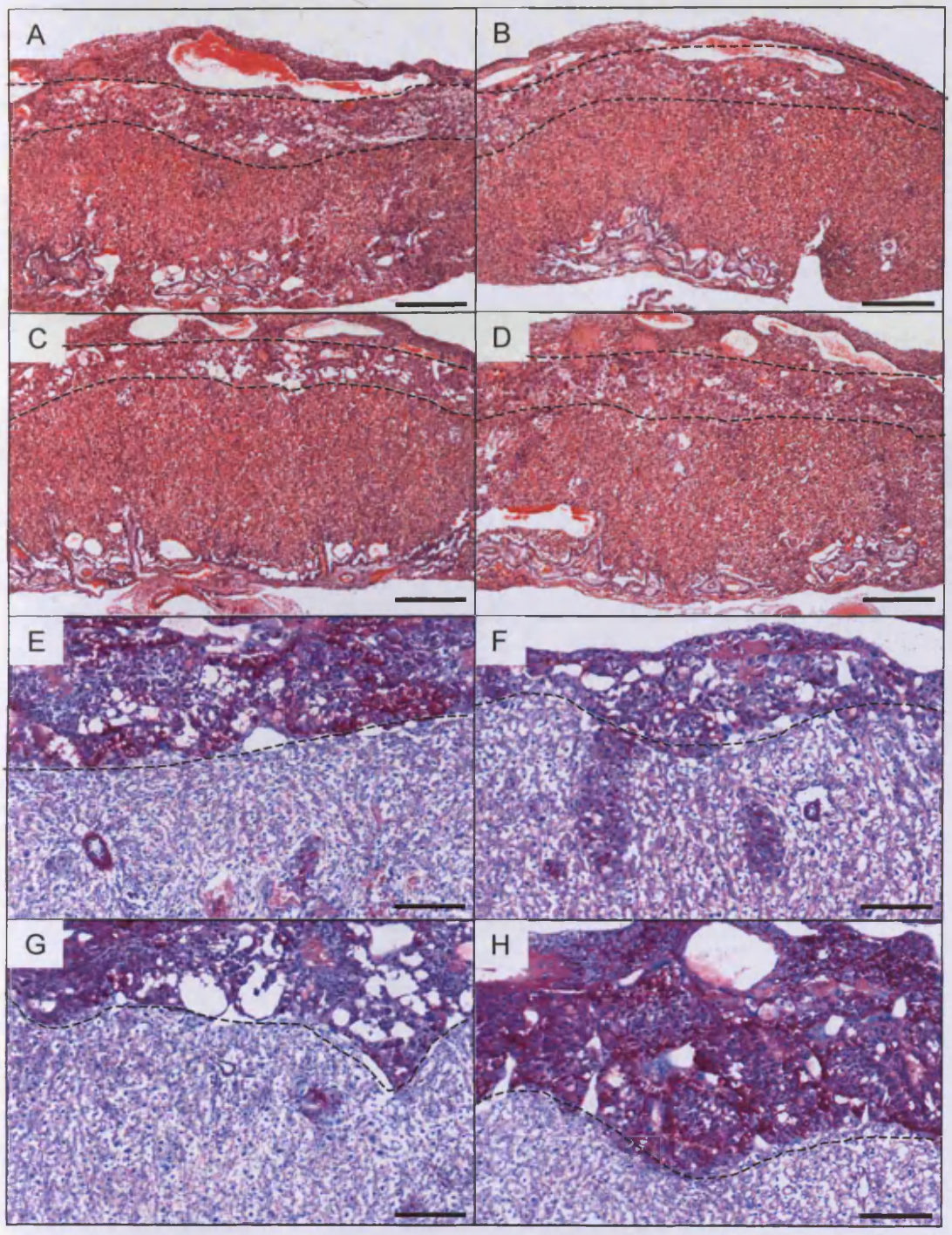


Figure 4.24: Glycogen staining at E18.5 in double transgenic matings

Placental morphology was examined from H&E stained sections at E18.5 (upper panels) and glycogen cells detected by PAS staining (lower panels). A and E) Wild type, B and F) 5D3 transgenic, C and G) 5D3 transgenic and *Phlda2* null, D and H) *Phlda2* null. Broken lines indicate boundaries between placental layers: labyrinth at bottom. Scale bar = 500 μm for A-D and 200 μm for E-H.

4.3 Discussion

There were three primary aims to the work presented in this chapter:

1. To confirm the key placental phenotypes of line 10-15 in an independent transgenic line
2. To determine if the placental phenotype associated with three additional copies of *Phlda2* and *Slc22a18* is affected by dosage of these genes
3. To dissect the relative contributions of *Phlda2* and *Slc22a18* to the placental phenotype

4.3.1 Confirmation of phenotype in an independent line

As transgenic males from the second transgenic line (10-10) were initially bred into the C57BL/6 genetic background, the placental phenotype of this line could only be examined on the 129/Sv x C57BL/6 background. To enable a direct comparison of the placental phenotype between lines 10-15 and 10-10, the phenotype of line 10-15 was first examined on the 129/Sv x C57BL/6 background. In addition to enabling a direct comparison of phenotypes between lines 10-15 and 10-10, this also provided an opportunity to assess the effect of genetic background on placental phenotype.

The placental phenotype of line 10-15 on the 129/Sv x C57BL/6 background at E14.5 was comparable to that of line 10-15 on a pure 129/Sv background. At this stage, transgenic placentae were ~20% lighter due to the disproportionate loss of spongiotrophoblast area, including an overt reduction in glycogen cell staining. No compensatory weight gain was observed in transgenic placentae on the 129/Sv x C57BL/6 background between E14.5 and E18.5. At E18.5, transgenic placentae on the pure 129/Sv background displayed a ~50% restoration of the weight towards the wild type mean, with a weight deficit of ~9% persisting to this stage. In contrast, the ~20% weight deficit of transgenic placentae on the 129/Sv x C57BL/6 background persisted until E18.5. A striking mislocalisation of *Tpbpa*-positive cells was observed in transgenic placentae on a 129/Sv background at E18.5, with a distinct spongiotrophoblast boundary absent at this stage. This phenotype was somewhat less severe on the 129/Sv x C57BL/6 genetic background, with a clear spongiotrophoblast-labyrinth border observed at E18.5. Occasional *Tpbpa*-positive cells were observed within the

labyrinth of transgenic placentae at both E14.5 and E18.5, although to a lesser extent than in placentae on a pure 129/Sv background. Additionally, *Tpbpa*-positive cells were observed in the labyrinth of wild type 129/Sv x C57BL/6 placentae at E14.5, although this appeared to occur at a lower frequency than in transgenic placentae, and was not observed in wild type placentae at E18.5. In contrast, *Tpbpa* positive cells were not observed to this extent in the labyrinth of wild type placenta on a pure 129/Sv background. This may represent another genetic background effect, with *Tpbpa* positive cells previously observed in the labyrinth of wild type placenta on an ICR x 129/Sv genetic background. However, in this same genetic background, placentae lacking *Parp1* expression exhibit a reduced spongiotrophoblast layer with an absence of *Tpbpa* positive cells in the labyrinth layer (Hemberger *et al.* 2003). This contrasts with this study in which mutant placenta displayed increased *Tpbpa* expression in distinct clusters of the labyrinth layer. Similar to transgenic placentae on a pure 129/Sv background, glycogen cell staining was visibly reduced at both E14.5 and E18.5 in placentae on the 129/Sv x C57BL/6 background. Additionally, glycogen staining did not co-localise with *Tpbpa*-positive cell clusters in the labyrinth in either wild type or transgenic placentae.

Despite the small variations in the phenotype described above, the placental consequences of over-expressing *Phlda2* and *Slc22a18* were comparable between a pure 129/Sv background and the 129/Sv x C57BL/6 background. The phenotype initially appeared to be more severe on the 129/Sv x C57BL/6 background, with the placental weight deficit persisting until term. However, histological examination of placental structure revealed that the mislocalisation of spongiotrophoblast was less severe in comparison to the 129/Sv placentae. These phenotypic variations underline the importance of characterising line 10-15 on the 129/Sv x C57BL/6 background in order to allow a direct comparison with line 10-10, which could only be investigated on the 129/Sv x C57BL/6 background.

Line 10-10 was generated during the course of this investigation and was initially bred into the C57BL/6 genetic background. Backcrossing of this line to the 129/Sv background has commenced, however due to time restraints, only transgenic animals on a C57BL/6 background were available to begin the characterisation of the placental phenotype. The transgene copy number of line

10-15 was previously determined by Southern blotting, and although this is a long-term aim for 10-10, it was not deemed necessary to accurately determine transgene copy number in this way. Instead, given the apparently stable expression of the transgene in line 10-15 on the 129/Sv x C57BL/6 background as determined by qPCR, this technique was also utilised to estimate copy number in line 10-10. Data from a comparison of two wild type and two transgenic placentae from the same litter suggest that line 10-10 contains 1-2 copies of the transgene. The primary objective for line 10-10 was to provide an independent transgenic line possessing the same transgene as line 10-15 in which to confirm a causative role for the genes located on the transgene. Further to this, line 10-10 additionally provided a line with an alternative transgene copy number to line 10-15 in which gene dosage effects could be investigated, which is discussed in Section 4.3.3.

The placental phenotype of line 10-10 was comparable to that of line 10-15 on the 129/Sv x C57BL/6 background. Transgenic placentae were ~20% lighter at E14.5, a deficit that persisted until E18.5, similar to line 10-15 on the 129/Sv x C57BL/6 genetic background. Furthermore, as for line 10-15 on the 129/Sv x C57BL/6 background, a distinct spongiotrophoblast boundary existed at both stages examined. Occasional *Tpbpa*-positive clusters of cells were observed in the labyrinth of transgenic placentae at E18.5, with varying frequency of these cells at E14.5 in wild type and transgenic placentae. The placental phenotypes of lines 10-15 and 10-10 were largely equivalent when examined on a similar 129/Sv x C57BL/6 background.

The development of similar placental phenotypes in two independently generated transgenic lines carrying the same transgene effectively excluded the possibility that the placental defect was a consequence of the site of transgene integration. A causative role for the site of transgene integration was considered an unlikely contributory factor to the placental phenotype given the previously reported results directly implicating *Phlda2*. Firstly, the phenotype of line 10-15 was reciprocal to that of the *Phlda2* null line, in which placentomegaly occurs due to an expansion of the spongiotrophoblast. Furthermore, the placental weight deficit and loss of spongiotrophoblast associated with *Kvdmr1*$^{+/-}$ placentae was rescued by restoring *Phlda2* gene dosage to normal in *Kvdmr1*$^{+/-}$ / *Phlda2*$^{-/+}$ double transgenic conceptuses (Salas *et al.* 2004). Nonetheless, the presence of

comparable phenotypes in two independent lines demonstrates a causative role for *Phlda2* and/or *Slc22a18*.

A striking visual difference was observed between wild type 129/Sv placentae and wild type placentae on the 129/Sv x C57BL/6 background. Those on a pure 129/Sv background appeared to have a smaller diameter and were more robust to handle prior to fixation. Wild type placentae from 129/Sv x C57BL/6 matings appeared to have a larger diameter and were considerably less rigid when handled prior to fixation. These initial visual characterisations of the two placentae prompted a comparison of weight and area data collected from wild type placentae on the two genetic backgrounds. At both E14.5 and E18.5, wild type placentae on the 129/Sv x C57BL/6 background were around ~40% heavier in comparison to those on a pure 129/Sv background. This increased weight was apparently due entirely to the presence of a larger spongiotrophoblast area in placentae on the 129/Sv x C57BL/6 background. The midline area of the spongiotrophoblast of these placentae was ~2.5 times greater than that seen in pure 129/Sv placentae, with no overt difference in the cross-sectional area of the labyrinth. Interestingly, perhaps due to the inherently larger spongiotrophoblast area of placentae on the 129/Sv x C57BL/6 background, transgenic placenta on this background possessed a larger spongiotrophoblast midline area than wild type placenta on a pure 129/Sv background (1.35 ± 0.40 versus 1.77 ± 0.45; n = 36; p = 0.0122). A summary of the mean midline spongiotrophoblast area is presented in Table 4.3.

To our knowledge, the striking structural differences between *Mus musculus* placentae on different genetic backgrounds discussed above have not been previously described, although F_1 hybrid placentae of a C57BL/6 x C3H cross were ~20% heavier than those of an inbred C57BL/6 cross (McLaren 1965). However, genetic background is known to impact upon phenotype severity, as demonstrated by the increased placentomegaly associated with *Phlda2* null mice on a pure C57BL/6 background compared with those on a C57BL/6 x 129/Sv background (Frank *et al.* 2002). The underlying cause of such genetic background effects remains uncertain. An awareness of the effect that genetic background may have on placental phenotype will be an important consideration for future investigation of the mouse placenta.

	WT area (mm^2)	Tg area (mm^2)	*P*
10-15 (129/Sv)	1.35 ± 0.40	0.54 ± 0.13	-
10-15 (129/Sv x C57BL/6)	3.17 ± 0.76	1.81 ± 0.42	0.0153
10-10 (129/Sv x C57BL/6)	3.17 ± 0.54	1.74 ± 0.49	0.0497
Combined 10-10 and 10-15 (129/Sv x C57BL/6)	3.17 ± 0.61	1.77 ± 0.45	0.0122

Table 4.4: Comparison of spongiotrophoblast midline area according to genetic background

Mean spongiotrophoblast midline area is presented for wild type and transgenic placenta of line 10-15 on a pure 129/Sv and the 129/Sv x C57BL/6 genetic backgrounds, line 10-10 on the 129/Sv x C57BL/6 genetic background, and the combined data for lines 10-15 and 10-10 on the 129/Sv x C57BL/6 background. Transgenic placentae of lines 10-15 and 10-10 on the 129/Sv x C57BL/6 background possess a larger midline spongiotrophoblast area than wild type placentae on a pure 129/Sv genetic background. The *p* value according to the Student's *t*-test represents a comparison between area on a pure 129/Sv background and area on the 129/Sv x C57BL/6 background.

Furthermore, abnormal placental development has been described in interspecific hybrids between different species of the genus *Mus*. For instance, hyperplasia was observed in placenta of a *Mus spretus* x *Mus musculus* cross, whereas the reciprocal *Mus musculus* x *Mus spretus* cross was associated with placental hypoplasia. Placental hyperplasia in these crosses was characterised by an expanded and disrupted spongiotrophoblast layer, whereas hypotrophic placenta were characterised by a reduction or complete loss of this layer (Zechner *et al.* 1996). The paternally expressed *Igf2* and the X-linked imprinted gene *Esx1* have been implicated in the placental dysplasia observed in interspecific hybrids (Zechner *et al.* 2002).

4.3.3 Effect of gene dosage on phenotype

Initial attempts to investigate the effect of gene dosage on phenotype focused on attempting to generate homozygous transgenic conceptuses of line 10-15 by mating hemizygous males with hemizygous females. Eleven litters were obtained at E14.5, comprising 19 wild type and 57 transgenic conceptuses, of which 7 were dying and an additional 20 conceptuses were at various stages of resorption. Although no statistical analysis was performed, the level of embryonic lethality observed was greater than seen in hemizygous matings with wild type

females, and thus it was decided not to proceed with this line of investigation. Instead, the alternative transgenic lines 10-10, 5D3 and 5A4 were used to examine the effect of gene dosage.

Analysis of gene expression by qPCR indicated the presence of 1-2 copies of the transgene in line 10-10. However, as discussed previously the placental phenotype of line 10-10 was comparable to that of line 10-15 (3 copies) on a similar genetic background, suggesting that dosage of *Phlda2* and *Slc22a18* did not impact the severity of the phenotype. To further investigate the effect of dosage on the phenotype a further two transgenic lines that possess similar transgenes to lines 10-15 and 10-10 were characterised. Lines 5D3 and 5A4 possess one and two copies of an unmodified BAC transgene respectively. The *Cdkn1c* gene is intact on these transgenes, although it is expressed only in a subset of embryonic tissues, with no placental expression from the transgene (John *et al.* 2001). Both lines were investigated on a similar 129/Sv x C57BL/6 genetic background to lines 10-15 and 10-10.

The placental phenotype observed in line 5D3 was similar to that seen in lines 10-15 and 10-10, with transgenic placentae ~27% lighter at E14.5 and ~23% lighter at E18.5. This placental weight deficit could be attributed to a disproportionate loss of the spongiotrophoblast layer. However, the extent of spongiotrophoblast loss in line 5D3 was more severe than in lines 10-15 and 10-10 on the 129/Sv x C57BL/6 background, with a 60% reduction in spongiotrophoblast area in transgenic placentae of line 5D3, compared to a ~45% reduction in area of lines 10-15 and 10-10. Furthermore, transgenic placentae of line 5A4 were ~40% lighter at E14.5 and ~30% lighter at E18.5, suggesting that the placental weight deficit in a transgenic line possessing two copies of an unmodified transgene is more severe than that of a line possessing a single copy of the same transgene.

There are two potential explanations to account for the increased severity of the placental phenotype observed in line 5A4 and to some extent in line 5D3. One possible explanation is that expression of the transgene is not copy number dependent in lines 5D3 and 5A4, with expression of *Phlda2* and *Slc22a18* actually greater than the two or three-fold wild type levels that would be expected in lines 5D3 and 5A4 respectively. Indeed, expression of these genes in line 5D3 on the 129/Sv x C57BL/6 genetic background was more than twice the

anticipated level. Thus, the more severe placental phenotype could be directly attributed to elevated *Phlda2* and *Slc22a18* expression. However, this seems unlikely, as the phenotype of line 10-15 has remained relatively constant despite initial reports of greater than 10-fold endogenous levels of *Phlda2* and *Slc22a18* (Salas *et al.* 2004).

An alternative explanation for the more severe placental phenotype seen in lines 5A4 and 5D3 is to consider the effect of *Cdkn1c* over-expression in the embryo. As a consequence of excess *Cdkn1c* in a subset of tissues, transgenic embryos from lines 5D3 and 5A4 were intrinsically growth restricted in a dosage dependent manner (Andrews *et al.* 2007). It can be presumed that such inherently smaller embryos place less demand on the placenta compared to wild type embryos. Consequently, the placenta of transgenic embryos from lines 5D3 and 5A4 would not need to be as large as normal in order to deliver the nutrients required for the embryos to reach their genetic growth potential. In contrast, embryos from lines 10-15 and 10-10 can be considered to have a normal growth potential and thus place greater demand on their associated placentae. The consequences of this scenario are addressed in Chapter 5. It is possible that the transgenic placenta of lines 5D3 and 5A4 in which *Phlda2* and *Slc22a18* are over-expressed would exceed the size required by the intrinsically small embryo. This relatively lower embryonic demand on the placenta may thus account for the more severe weight deficit of placentae from these lines and the greater reduction in spongiotrophoblast area observed. A potential method to investigate this theory would be to generate chimeric conceptuses comprising transgenic 5A4 or 5D3 embryos and wild type tetraploid embryos that contribute only to extraembryonic tissue (Nagy *et al.* 1990). In this way, if transgenic embryos directly limit the size of their associated placenta, then the mean weight of placentae from such chimeric animals would be significantly smaller than that of placentae from chimeric conceptuses consisting of wild type and tetraploid wild type cells. The potential for interaction between the embryo and placenta is discussed further in Chapter 5.

4.3.4 Attributing the phenotype to *Slc22a18* or *Phlda2*

The relative contribution of *Phlda2* to the placental phenotype was examined in a cross between the *Phlda2* null line and the single copy transgenic line 5D3. The

aim of this cross was to generate double transgenic conceptuses with a normal gene dosage of *Phlda2* but with two copies of *Slc22a18* and which could be compared to both wild type and 5D3 transgenic conceptuses. The basic principles of this experiment were similar to that described in Salas *et al.* (2004), in which the role of *Phlda2* in the *Kvdmr1* null phenotype was isolated by restoring *Phlda2* dosage to normal by crossing lines *Kvdmr1*$^{+/-}$ and *Phlda2*$^{-/+}$. A comparison between *Kvdmr*$^{+/-}$ and *Kvdmr1*$^{+/-}$ / *Phlda2*$^{-/+}$ placenta revealed that restoring *Phlda2* gene dosage to normal entirely rescued the spongiotrophoblast deficit with a 50% rescue of the weight deficit at both E14.5 and E16.5 (Salas *et al.* 2004). Interestingly, *Phlda2* mRNA levels were somewhat variable in both *Kvdmr*$^{+/-}$ and *Kvdmr1*$^{+/-}$ / *Phlda2*$^{-/+}$ placenta, with *Phlda2* expression apparently failing to completely normalise in some *Kvdmr1*$^{+/-}$ / *Phlda2*$^{-/+}$ placenta.

In this investigation, the mean placental weight of double transgenic placentae was restored to within the normal range at E14.5, with no significant difference existing between placental weight of wild type and double transgenic placentae. In contrast, only a 50% rescue in spongiotrophoblast area was achieved at this stage, with the area of double transgenic placentae significantly larger than that of 5D3 transgenic placentae but remaining significantly smaller than that of wild type. This could be because *Phlda2* gene expression was not restored to wild type levels. However, this seems unlikely, as data presented in this chapter suggests that gene dosage does not affect phenotype severity, with lines 10-15 and 10-10 exhibiting a comparable placental phenotype despite different levels of *Phlda2* expression. Furthermore, the phenotype of line 10-15 has remained comparable to the previously published report despite a ~2.5 fold reduction in *Phlda2* expression levels (Salas *et al.* 2004).

Alternatively, the reduced spongiotrophoblast area may be attributed to an effect of *Cdkn1c* over-expression in the embryo. Transgenic embryos of line 5D3 are intrinsically growth restricted due to excess embryonic *Cdkn1c* expression in a subset of tissues (Andrews *et al.* 2007). Additionally, embryos of the *Phlda2* null line used in this investigation were also reported to be 13% lighter than wild type at E16.5 (Frank *et al.* 2002). As described in Section 4.3.3, the extent of spongiotrophoblast loss in line 5D3 was more severe than observed in lines 10-15 and 10-10. One possible explanation for the reduced spongiotrophoblast of double transgenic placentae is that the growth potential of the embryo may be

linked to the volume and functional capacity of the placenta. Thus, restoring *Phlda2* gene dosage to normal would be unable to entirely rescue the spongiotrophoblast loss, with the remaining phenotype instead attributable to the effect of an intrinsically small embryo exerting reduced demand on the placenta.

One way in which to determine whether excess placental *Phlda2* is causative of the remaining spongiotrophoblast deficit would be to measure spongiotrophoblast area only from placenta in which *Phlda2* expression is restored to wild type levels. This would be achieved by determining gene expression and spongiotrophoblast area from opposite halves of the same placenta. This approach assumes that *Phlda2* expression will be restored to wild type levels in some placenta. However, this assumption seems reasonable given that *Phlda2* expression is variable even in wild type placenta, with expression levels in individual placenta previously observed to range between 0.5 and 1.5 times the mean wild type level (Salas *et al.* 2004).

Although these results do not fully exclude a role for excess *Slc22a18* in the placental phenotype described, it seems unlikely that *Slc22a18* plays a significant causative role for several reasons. Firstly, the phenotype of placenta that over-express *Phlda2* and *Slc22a18* is reciprocal to that observed in *Phlda2* null placenta, thus solely implicating a role for *Phlda2*. Secondly, the *Phlda2* and *Slc22a18* gene products have distinct functions, with Slc22a18 being a putative organic cation transporter (Dao *et al.* 1998), and Phlda2 implicated in phosphatidyl inositol phosphate signalling (Frank *et al.* 2002; Saxena *et al.* 2002). These diverse functions do not implicate excess *Slc22a18* in altering placental structure or spongiotrophoblast size. Thirdly, introduction of the targeted *Phlda2* null allele to the single copy BAC line is able to restore an almost normal phenotype. Confidently excluding a role for *Slc22a188* in the described placental defect will require the generation of new transgenic lines in which either *Phlda2* or *Slc22a18* are over-expressed in isolation.

4.3.5 Summary of main findings

Two of the three primary objectives to this work were achieved in full. The independent transgenic line 10-10 was characterised at two key stages, demonstrating a comparable phenotype to line 10-15, and thus confirming a causative role for *Phlda2* and/or *Slc22a18* in the placental phenotype. The effect

of gene dosage was investigated in four transgenic lines possessing between one and three copies of either a modified or unmodified transgene. Similar placental phenotypes were observed in transgenic lines possessing a single copy of the unmodified transgene and lines possessing two or three copies of the modified transgene demonstrating that there was no overt gene dosage effect on phenotype severity. Furthermore, these results indicate that lines 10-15 and 10-10 may be used as a model for LOI of *Phlda2* and *Slc22a18*. This will prove particularly useful in embryonic and post-natal investigations due to the absence of excess *Cdkn1c* that persists in line 5D3.

To attempt to dissect the relative contributions of *Phlda2* and *Slc22a18* to the placental phenotype, the single copy transgenic line was crossed with the *Phlda2*$^{-/+}$ line to generate conceptuses possessing a single active copy of *Phlda2* but maintaining two active copies of *Slc22a18*. However, despite the presence of only one active copy of *Phlda2*, expression level of this gene failed to normalise in such conceptuses. It was therefore not possible to entirely exclude a role for *Slc22a18* in the placental phenotype, although as discussed above it seems unlikely that both *Phlda2* and *Slc22a18* would have similar functions. Potential approaches for examining the contribution of *Slc22a18* are discussed below.

4.3.6 Future directions

A number of opportunities exist for pursuing further work based upon the findings reported in this chapter. A clear effect of genetic background on the phenotype was demonstrated between placentae on a pure 129/Sv background and those on the 129/Sv x C57BL/6 background. Line 10-10 is currently being bred onto the 129/Sv background with the intention of characterising the phenotype at E14.5 and E18.5, enabling a future comparison with line 10-15 on a pure 129/Sv background. Further interest may also lie in characterising the placental phenotype on a pure C57BL/6 background. This is due to the intended future use of adult mice on a pure C57BL/6 background in post-natal studies. To complement the characterisation of the above transgenic lines on both pure 129/Sv and 129/Sv x C57BL/6 genetic backgrounds, future work could also focus entirely on describing the development and structure of the mouse placenta across different genetic backgrounds. For example, Coan *et al.* (2004)

characterised placental development in the pure C57BL/6 strain, although a comparison with other genetic backgrounds was not performed.

An important aspect remains unresolved in that a role for *Slc22a18* in the placental phenotype could not be entirely excluded based on the results obtained. The failure to restore *Phlda2* expression to wild type levels may reflect the variable expression of *Phlda2* from the transgene (Salas *et al.* 2004). This persistent expression of *Phlda2* above the anticipated level may be restored following further generations of breeding line 5D3 onto the C57BL/6 background. Thus, additional material will be generated using transgenic 5D3 animals that have been bred further into the C57BL/6 background. After weighing, each placenta will be cut approximately in half, with one side of the placenta used for RNA isolation and the other side fixed and paraffin embedded for sectioning. Following genotyping, *Phlda2* expression will be examined and only double transgenic placenta in which ΔC_T values are restored to wild type levels will be analysed. This is clearly a more labour intensive and time-consuming approach, and assumes that the failure to rescue the phenotype can be attributed to variable *Phlda2* levels in double transgenic placenta. A more direct approach would be to generate a new transgenic line, of which several possibilities exist. The existing transgene could be modified to disrupt either *Phlda2* or *Slc22a18* thus enabling the effect of over-expressing only one of these genes to be examined in isolation. The preferred option would be to target *Slc22a18* thus creating a transgenic line in which the placental, embryonic and post-natal consequences of over-expression of *Phlda2* could be studied. A third potential option would be to create an *Slc22a18* null model. This model would reveal *Slc22a18* gene function, however it would not provide a tool in which to examine the imprinting of this gene.

Finally, and perhaps most intriguingly, data presented in this chapter could suggest an interaction between *Cdkn1c* dosage in the embryo and *Phlda2* dosage in the placenta in order to achieve a balance between embryonic demand and placental supply of nutrients. Generation of a transgenic line possessing a single copy of the modified BAC transgene would enable a direct comparison with line 5D3 and further investigation of this hypothesis.

Chapter 5:

Embryonic and post-natal consequences of excess *Phlda2* and *Slc22a18*

5.1 Overview

Work presented in chapters 3 and 4 characterised the placental defects associated with increased dosage of *Phlda2* and *Slc22a18*. The consequence of such over-expression was a significant reduction in placental weight due to the restricted expansion of the spongiotrophoblast layer. Initial results indicated that embryonic growth on a mixed genetic background remained normal until E14.5 but was significantly impaired at E16.5 (Salas *et al.* 2004). The placental defect and associated late gestation embryonic growth deficit is consistent with placental insufficiency negatively restricting growth of the embryo.

In the Western world, placental insufficiency is predicted to be the primary causative factor of IUGR (Henriksen and Clausen 2002), affecting up to 7% of all births (Brodsky and Christou 2004; Vandenbosche and Kirchner 1998). Placental insufficiency is generally associated with asymmetric IUGR with the growth-restricting factor affecting growth during the third trimester (Lin and Santolaya-Forgas 1998; Sifianou 2006). Approximately 70-80% of IUGR cases are classified as asymmetric, characterised by an increased head circumference to abdominal circumference (HC/AC) ratio. This disproportionate growth restriction is a consequence of the relative sparing of brain and skeletal development at the expense of liver and subcutaneous fat development (reviewed in Fay and Ellwood 1993; Platz and Newman 2008; Valsamakis *et al.* 2006). The immediate risk of asymmetric IUGR is a significantly increased risk of perinatal mortality (Fay and Ellwood 1993; Kok *et al.* 1998; McIntire *et al.* 1999; Sifianou 2006). However, individuals that survive the neonatal period exhibit catch-up growth, which is not generally observed in infants with symmetric IUGR, with these individuals remaining small throughout life (Fay and Ellwood 1993).

Numerous epidemiological studies in humans have reported a correlation between low birth weight and the development of metabolic syndrome in adulthood. Low birth weight has been associated with increased rates of CVD (Barker *et al.* 1993; Barker and Osmond 1986; Osmond *et al.* 1993) hypertension (Barker *et al.* 1990; Law *et al.* 1993) and impaired glucose tolerance (Hales *et al.* 1991). The association of birth weight with obesity tends to follow a U-shaped relation, with both low and high birth weights associated with increased rates of adult obesity. Contrastingly, high birth weight tends to predict an increased body

mass index (BMI) in adulthood, whereas low birth weight is specifically associated with central obesity (Parsons *et al.* 2001; Ravelli *et al.* 1999; Te Velde *et al.* 2003). Furthermore, catch-up growth has been identified as an additional contributory factor, with low birth weight infants that displayed catch up growth by two years of age more likely to be obese at five years of age (Ong *et al.* 2000). Indeed, the risk of developing other aspects of metabolic syndrome was increased in individuals with a low birth and subsequent post-natal catch up growth (Eriksson *et al.* 2000; Eriksson *et al.* 1999; Forsen *et al.* 2000; Forsen *et al.* 1999).

The "developmental origins of adult disease" hypothesis proposes that the risk of developing aspects of metabolic syndrome is modified by poor growth *in utero* manifesting as a low birth weight (Barker 1995; Hales and Barker 1992). This predisposition to development of disease in adulthood is proposed to occur through "fetal programming", whereby an undernourished fetus adapts to a low nutrient environment *in utero*. These adaptations irreversibly alter the metabolism, subsequently resulting in a mismatch between the perceived low nutrient environment predicted *in utero*, and the actual nutrient rich environment. The altered metabolism is thus unable to adapt to the nutrient rich environment causing a predisposition to metabolic syndrome (Barker 1997). It has been proposed that there are two critical windows involved in the programming of metabolic syndrome. Poor *in utero* growth followed by rapid postnatal catch up growth are proposed to represent the greatest risk, with prevention of catch-up growth affording some protection (reviewed in Jimenez-Chillaron and Patti 2007).

The initial association between low birth weight and metabolic disease was identified from human epidemiological studies. However, such studies are inherently long-term, and provide little insight into the underlying mechanisms. Animal models provide useful tools for elucidating the mechanisms that may underlie fetal programming associated with IUGR. Indeed, a number of animal models of IUGR have been developed that are associated with various aspects of metabolic syndrome in adulthood. One of the most extensively studied rodent models of IUGR is the maternal low protein model. Feeding pregnant rats a low protein diet during gestation and lactation predisposes animals to developing insulin resistance (Fernandez-Twinn *et al.* 2005; Petry *et al.* 2001). Similarly, calorie restriction of pregnant rats from day 10 of gestation resulted in IUGR with

catch up growth displayed by animals suckled by *ad libitum* fed dams. These animals became heavier than control animals with increased fat mass and reduced lean mass at 9 months of age. However, in this instance, continued exposure to food restriction during the lactation period delayed catch up growth and prevented the alteration of lean and fat mass proportions (Desai *et al.* 2005). Additionally, calorie restriction during the last week of gestation in mice was associated with post-natal catch up growth, glucose intolerance and β-cell dysfunction (Jimenez-Chillaron *et al.* 2005). Continuation of calorie restriction during lactation protected against glucose intolerance (Jimenez-Chillaron *et al.* 2006). However, a genetic model of IUGR that recapitulates known conditions in human patients has not been reported to date.

Data presented in Chapters 3 and 4 described the placental defect associated with over-expression of *Phlda2* and *Slc22a18* in the mouse placenta. Recent reports from human studies have described elevated levels of *PHLDA2* in the placentae of IUGR and lower birth weight infants (Apostolidou *et al.* 2007; McMinn *et al.* 2006). Transgenic embryos on a mixed genetic background displayed reduced embryonic growth at E16.5 (Salas *et al.* 2004), although no weight difference was observed at 3 weeks of age (Andrews *et al.* 2007). Embryonic growth of line 10-15 was further characterised on a pure 129/Sv genetic background to determine if similar growth restriction was observed on this genetic background. Similarly, embryonic growth was examined in lines 10-15 and 10-10 on the 129/Sv x C57BL/6 background to assess the effect of genetic background on embryonic growth.

As described above, placental insufficiency is associated with asymmetric IUGR, which is similarly associated with post-natal catch up growth and a predisposition to metabolic syndrome. Further work was performed to ascertain whether the placental defect of line 10-15 was responsible for the reported IUGR, and whether this was associated with metabolic syndrome in adult animals. This characterisation included the examination of placental transporter expression levels to determine if transgenic placenta displayed a compensatory increase in transport potential, which would be indicative of placental insufficiency. Two key features of metabolic syndrome, reduced glucose tolerance and increased adiposity, were examined in adult animals.

5.2 Results

5.2.1 The placental defect is linked to embryonic growth restriction

Previous work reported that at E14.5, transgenic embryos were slightly larger by weight, although without achieving statistical significance, whereas at E16.5, transgenic embryos were 10% lighter, but with only marginal statistical significance (Salas *et al.* 2004). The effect of excess *Phlda2* and *Slc22a18* on embryonic growth was further investigated by generating additional litters at these time-points in addition to earlier and later stages in development on a pure 129/Sv genetic background. At least six litters with a minimum of two transgenic and two wild type animals each were collected at each developmental stage. Raw mean wet weights of wild type and transgenic placentae are presented in Figure 5.1.

At E12.5, transgenic embryos weighed approximately 9% less than wild type, with marginal statistical significance (76.0 mg ± 11.6 versus 69.3 mg ± 12.6; 9 litters; n = 74; p = 0.019). At E13.5 transgenic embryos were no different by weight compared to wild type (124.9 mg ± 16.2 versus 125.7 mg ± 10.1; 6 litters; n = 41; p = 0.846). At E14.5, transgenic embryos were 3.6% lighter, although again without statistical significance (222.5 mg ± 35.4 versus 214.6 mg ± 19.9; 7 litters; n = 53; p = 0.346). Transgenic embryos at E16.5 were 5.8% lighter (600.0 mg ± 63.5 versus 565.1 mg ± 50.3; 8 litters; n = 58; $p = 2.48 \times 10^{-2}$). Further to previous work, embryonic weights were also examined at E18.5, representing the most advanced stage that can be characterised prior to birth. At this stage, transgenic embryos were found to be 11.1% lighter than wild type animals (1212.8 mg ± 143.4 versus 1078.0 mg ± 149.2; 10 litters; n = 68; $p = 6.16 \times 10^{-4}$). At all gestational stages examined, wild type and transgenic conceptuses were observed at the expected frequencies (see Section 3.2.2 for χ^2 values).

Individual raw weights of wild type embryos exhibited a standard deviation of up to 18% the mean weight for each gestational stage. Such variations in weight may be caused by a number of factors that could not be controlled for, such as litter size, exact gestational age, position of the conceptus in the uterine horn, maternal health and other environmental factors. To prevent such factors from potentially disguising or introducing apparent growth phenotypes, individual weights of both wild type and transgenic embryos were adjusted by the ratio of

the mean wild type weight for the gestational stage to the mean wild type weight for the litter, as described in Section 2.19.4. Such manipulation did not alter the mean wild type weight, but adjusts the mean transgenic weight accordingly and reduces the standard deviation of both wild type and transgenic data sets. Mean normalised embryonic weights of wild type and transgenic embryos are presented in Fig 5.1B, with error bars representing standard deviation of normalised data. The average transgenic weight is also presented as a percentage of mean wild type weight for each gestational stage.

At E12.5, transgenic embryos weighed approximately 6% less than wild type, although without reaching statistical significance (76.0 mg ± 7.5 versus 71.6 mg ± 12.8; 9 litters; n = 74; $p = 7.13 \times 10^{-2}$). At E13.5 transgenic were no different by weight compared to wild type (124.9 mg ± 11.8 versus 126.4 mg ± 10.3; 6 litters; n = 41; $p = 0.664$). At E14.5, transgenic embryos were 2.8% lighter, again without statistical significance (222.5 mg ± 17.4 versus 216.2 mg ± 26.1; 7 litters; n = 53; $p = 0.293$). Transgenic embryos at E16.5 were 6.6% lighter (600.0 mg ± 48.3 versus 560.5 mg ± 40.3; 8 litters; n = 58; $p = 1.39 \times 10^{-3}$). At E18.5 transgenic embryos were 13.2% lighter than wild type animals (1226.8 mg ± 58.6 versus 1068.6 mg ± 88.6; 10 litters; n = 68; $p = 8.66 \times 10^{-15}$).

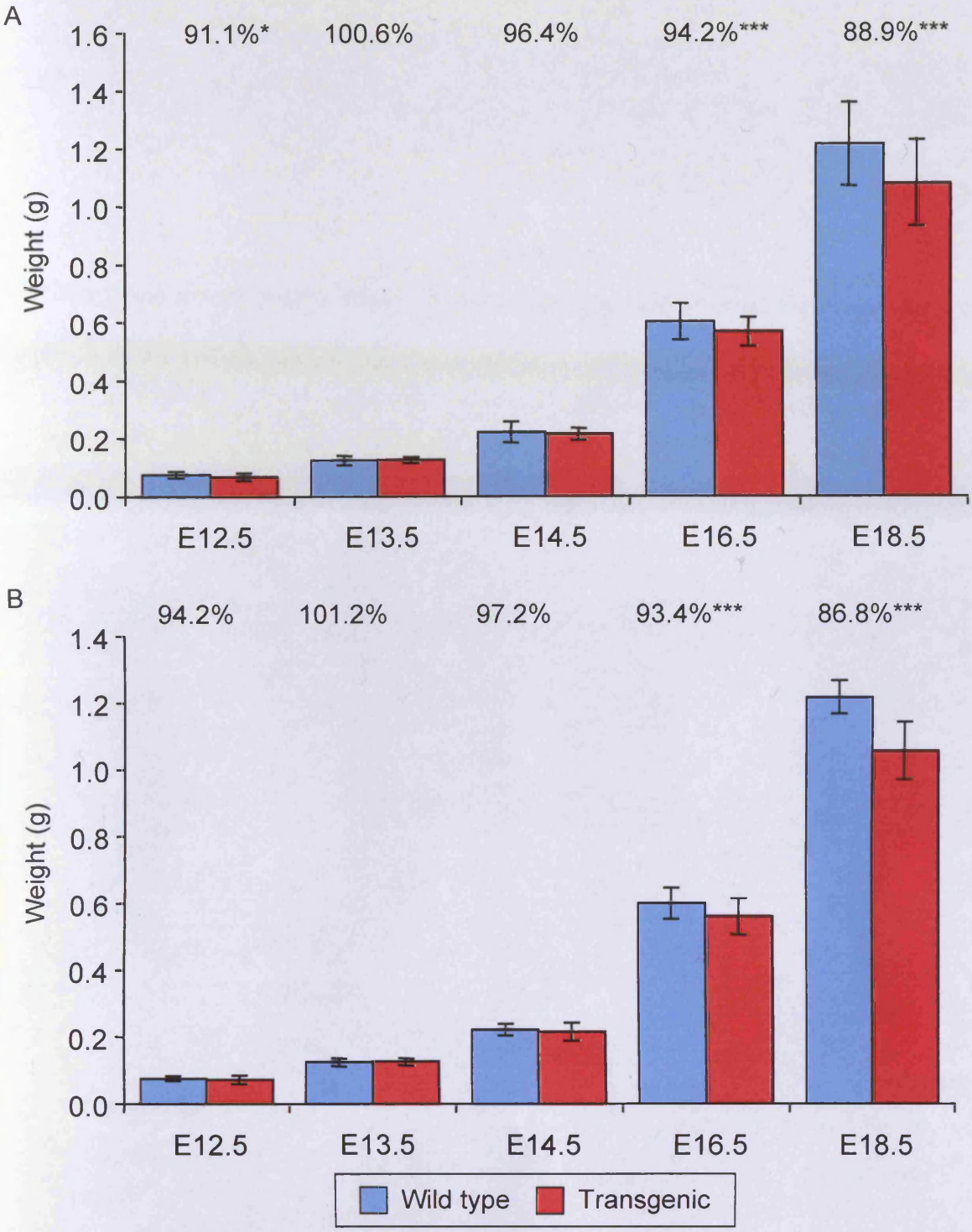


Figure 5.1: Weights of wild type and transgenic embryos from line 10-15 between E12.5 and E18.5

Graphs showing mean A) raw and B) normalised embryonic weights for wild type and transgenic embryos of line 10-15 on a pure 129/Sv background at the indicated gestational stages. Error bars show standard deviation. Percentage figures indicate the relative weight of transgenic embryos compared to wild type embryos at each stage. Significance level according to the Student's *t*-test indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Embryonic growth was also examined at E14.5 and E18.5 for embryos from line 10-15 on the 129/Sv x C57BL/6 genetic background, in addition to embryos from line 10-10 on a similar genetic background. Figure 5.2 shows the normalised mean weights of wild type and transgenic embryos from line 10-15 on a 129/Sv background, and from lines 10-15 and 10-10 on the 129/Sv x C57BL/6 background at E14.5 and E18.5. At E14.5, transgenic embryos from line 10-15 on a pure 129/Sv background were 2.8% lighter, although without achieving statistical significance. Similarly, transgenic embryos from the same line on the 129/Sv x C57BL/6 genetic background were not significantly different in weight (261.7 mg ± 22.7 versus 259.8 mg ± 24.8; 4 litters; n = 35; $p = 0.822$). Transgenic embryos from line 10-10 were also no different by weight (241.2 mg ± 11.9 versus 241.9 mg ± 27.2; 6 litters; n = 46; $p = 0.909$). At E18.5, transgenic embryos from line 10-15 on a pure 129/Sv background were 13.2% lighter. In contrast, transgenic embryos of line 10-15 on the 129/Sv x C57BL/6 background were only 4.4% lighter than wild type (1327.6 mg ± 64.2 versus 1269.1 mg ± 86.9; 6 litters; n = 37; $p = 0.0278$), whereas those from line 10-10 were 5.4% lighter (1322.9 mg ± 57.7 versus 1246.2 mg ± 79.9; 4 litters; n = 26; $p = 0.0089$).

The growth restriction of transgenic embryos at E18.5 apparent on the 129/Sv x C57BL/6 background was less than half of that observed on the pure 129/Sv background. Interestingly, as observed for placentae, wild type embryos on the 129/Sv x C57BL/6 genetic background were significantly heavier than wild type embryos on a pure 129/Sv background at both gestational stages examined (Fig 5.3). At E14.5, wild type embryos on the 129/Sv x C57BL/6 background were 11.9% heavier than those on a pure 129/Sv background (222.5 mg ± 35.4 versus 249.0 mg ± 19.4; wild type weights from 19 litters; n = 73; $p = 1.13 \times 10^{-4}$), with a similar difference in weight of 8.5% at E18.5 (1215.7 mg ± 54.9 versus 1318.6 mg ± 60.7; wild type data from 22 litters; n = 82; $p = 6.57 \times 10^{-12}$).

In summary, embryonic weight of transgenic animals was significantly less than wild type littermates at E18.5 for line 10-15 on both a pure 129/Sv and 129/Sv x C57BL/6 genetic background and also for line 10-10. However, the extent of the weight deficit was more severe on a pure 129/Sv genetic background (~13%), with only a ~5% weight deficit observed for transgenic embryos of lines 10-15 and 10-10 on the 129/Sv x C57BL/6 background.

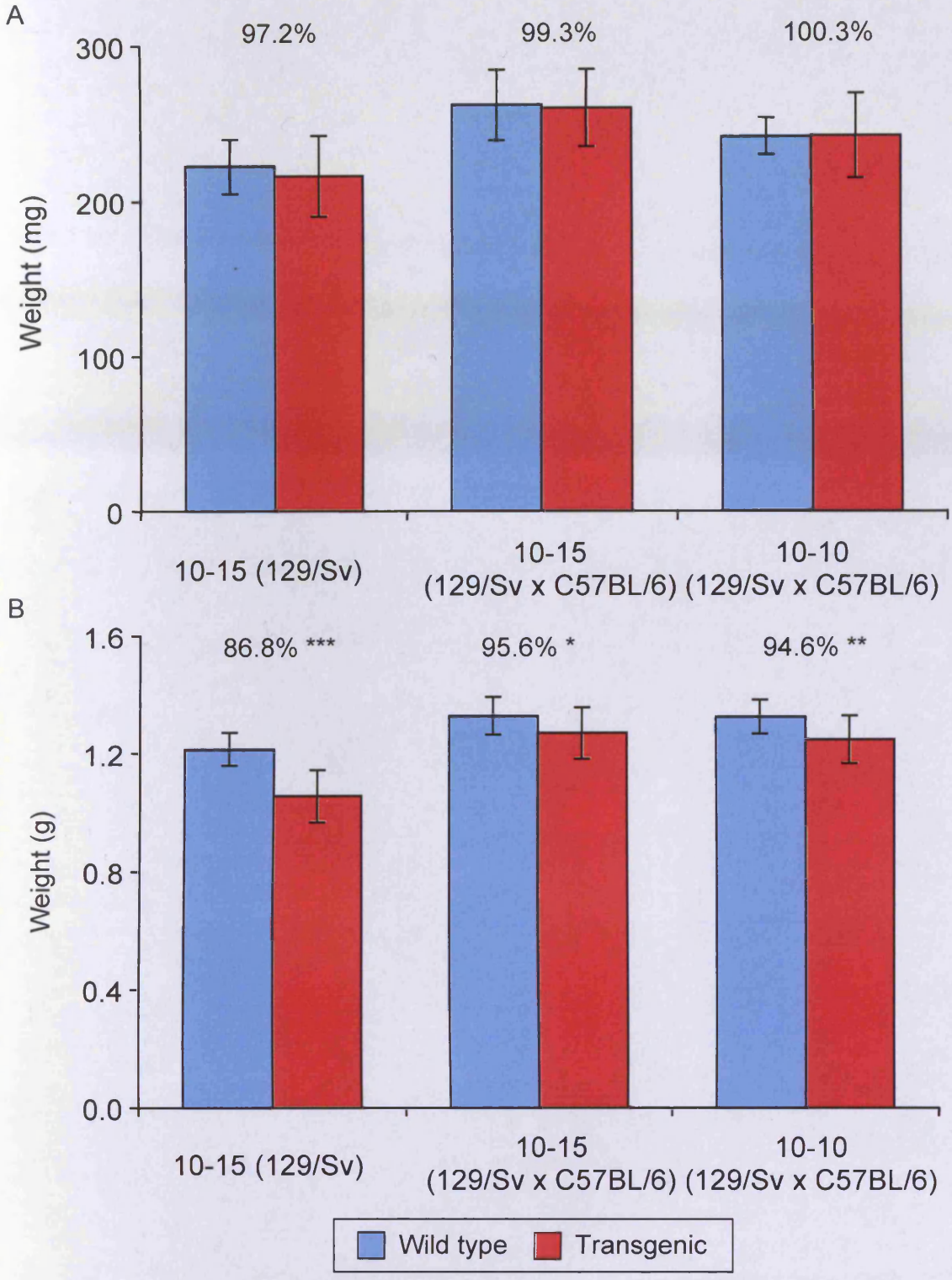


Figure 5.2: Embryonic growth in lines 10-15 and 10-10

Normalised mean weights of wild type and transgenic embryos are shown for lines 10-15 on a pure 129/Sv background and lines 10-15 and 10-10 on the 129/Sv x C57BL/6 background at A) E14.5 and B) E18.5. Percentage figures indicate the relative weight of transgenic embryos compared to wild type. Error bars show standard deviation; asterisks denote significance level according to the Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

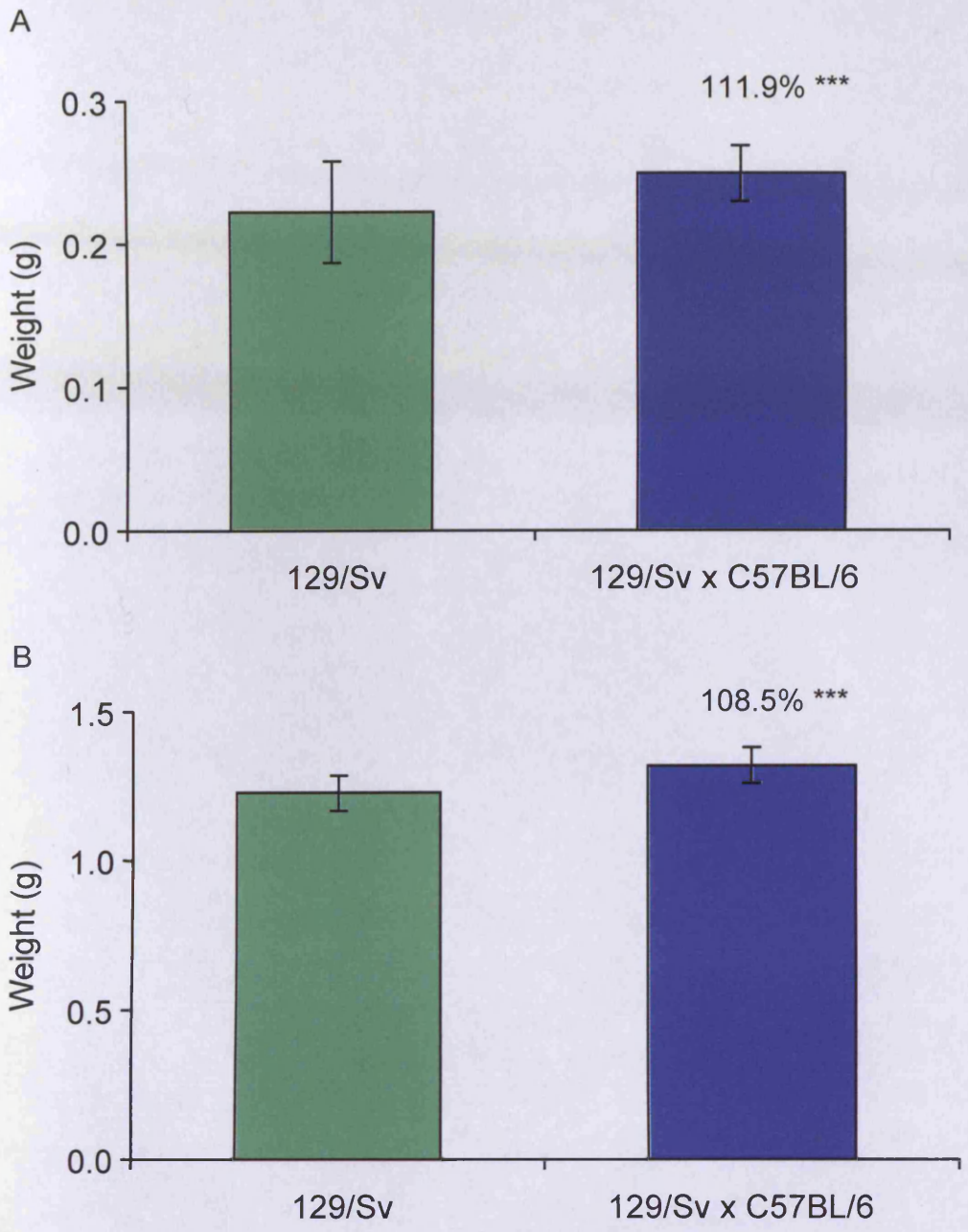


Figure 5.3: Comparison of wild type embryonic weights at E14.5 and E18.5 between pure 129/Sv and 129/Sv x C57BL/6 backgrounds

Normalised mean weights of wild type embryos are shown on the 129/Sv and 129/Sv x C57BL/6 genetic backgrounds at A) E14.5 and B) E18.5. Percentage figures indicate the ratio of mean embryonic weight on the 129/Sv x C57BL/6 background to that on the pure 129/Sv background. Error bars show standard deviation; asterisks denote significance level according to the Student's *t*-test: *** $p < 0.001$.

5.2.2 Transgenic animals exhibit rapid post-natal catch-up growth

The effect of over-expression of *Phlda2* on post-natal growth has not previously been directly investigated. However, a comparison between *Kvdmr1* null mice (all genes in the IC2 domain are over-expressed) and *Phlda2 / Kvdmr1* double null mice (*Phlda2* levels returned to normal), revealed a marginal increase in weight at P14 in *Phlda2 / Kvdmr1* double null animals, although this was not statistically significant (Salas *et al.* 2004). We recently reported that at three weeks of age, there was no significant difference in weight between transgenic and wild type animals of line 10-15 on a mixed genetic background (Andrews *et al.* 2007).

The extent of growth restriction immediately following birth and in the subsequent neo-natal period was investigated by the removal and sacrifice of entire litters on the day of birth and the following day; post-natal day 0 (P0) and P1 respectively. Data was normalised as described in Section 2.19.4, and further divided into groups according to the estimated time of birth. Litters that were born during the night were not discovered until 9 am, whereas some litters were born during daylight hours, and were removed immediately upon discovery. These groups are referred to as "P0 fed" and "P0 unfed" respectively, whereas "P0 combined" represents the pooled data of both groups. Mean normalised weights of transgenic and wild type animals for each group are presented in Figure 5.4, with error bars representing the standard deviation.

A substantial number of litters were obtained at P0, and were initially studied as a single set of data, with the birth weight of transgenic animals 11.3% less than wild type (1.41 g ± 0.08 versus 1.25 g ± 0.13; 22 litters; n = 154; p = 3.29×10^{-17}). In comparison, those litters that were born during the day, and thus had little time to begin feeding, the birth weight of transgenic animals was 13.1% less than wild type (1.39 g ± 0.5 versus 1.21 g ± 0.15; 7 litters; n = 63; $p = 5.14 \times 10^{-9}$). In contrast, transgenic animals from litters born during the night, which had thus begun feeding, were only 10.1% lighter than wild type (1.42 g ± 0.10 versus 1.28 g ± 0.11; 15 litters; n = 96; $p = 1.39 \times 10^{-9}$). By P1, the weight difference between transgenic and wild type animal weights was reduced to 7.3% (1.62 g ± 0.11 versus 1.51 g ± 0.11; 6 litters; n = 49; $p = 3.9 \times 10^{-4}$).

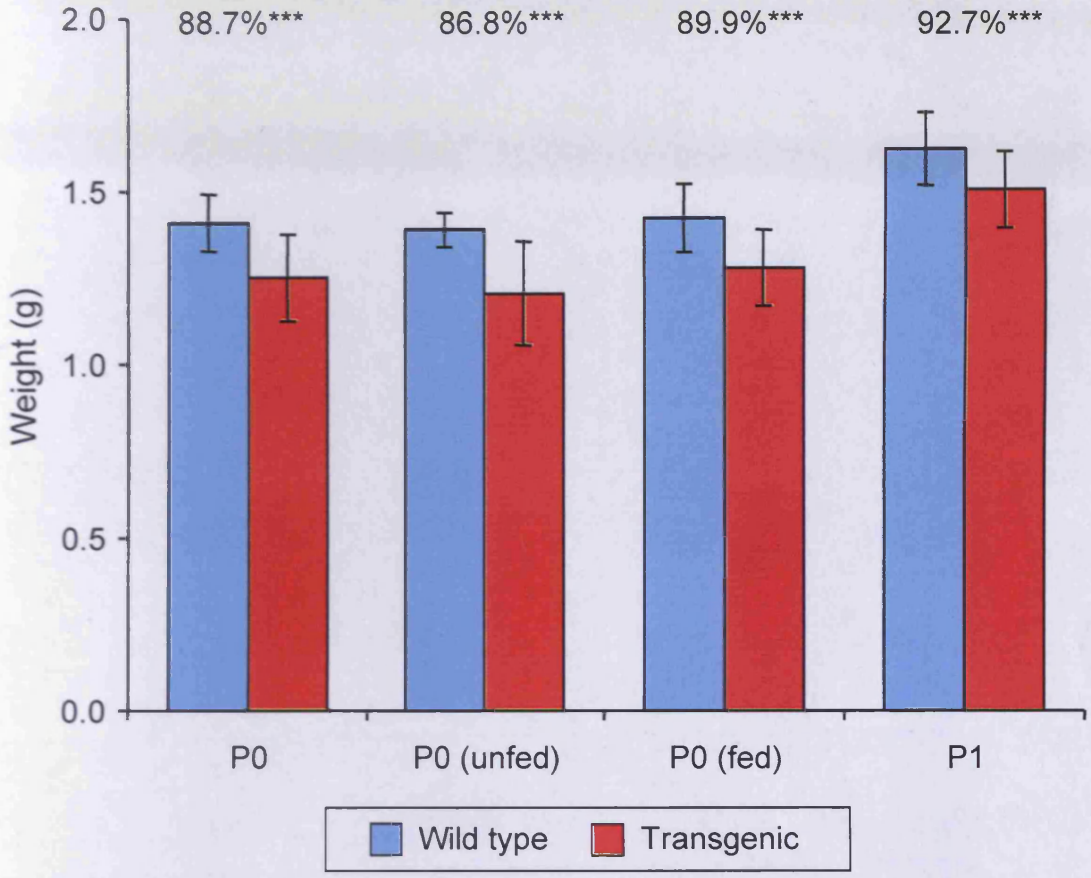


Figure 5.4: Neo-natal weights of mice from line 10-15

Mean normalised weights for wild type and transgenic embryos of line 10-15 on a pure 129/Sv background at P0 and P1. Data at P0 is divided according to time of birth, P0 fed (births during night), P0 unfed (births during day), and displayed collectively as P0. Error bars show standard deviation. Percentage figures indicate the relative weight of transgenic embryos compared to wild type embryos at each stage. Asterisks denote significance level according to the Student's *t*-test: *** $p < 0.001$.

The 13% difference between transgenic and wild type birth-weight was approximately halved to 7% within one day of birth. A cohort of animals was generated to examine growth over the course of a year from one week of age. All animals used in the weighing study were born within a two-week period and were weaned exactly 28 days following birth. A minimum litter size of 6 was accepted for the study, with littermates of the same sex co-housed after weaning. Animals of different litters were not mixed together, and no animals were housed alone. Mice from the cohort were not used for breeding and had *ad libitum* access to food and water.

Mean raw weights are presented in Figure 5.5 for animals up to weaning age. At one week of age, although transgenic animals appear to be approximately 5% lighter than wild type animals, there was no significant difference in weight for either male (3.57 g ± 0.52 versus 3.35 g ± 0.62; n = 47; p = 0.203) or female animals (3.47 g ± 0.62 versus 3.35 g ± 0.55; n = 57; p = 0.425). At two weeks of age, there was no discernable difference in weight for either males (6.51 g ± 0.85 versus 6.42 g ± 0.81; n = 69 p = 0.672) or females (6.40 g ± 0.92 versus 6.47 g ± 0.94; n = 78 p = 0.747). No significant difference in weight was detected for either male (8.07 g ± 1.40 versus 8.12 g ± 1.35; n = 69 p = 0.672) or female animals (8.05 g ± 1.43 versus 8.06 g ± 1.34; n = 78 p = 0.954) at three weeks of age. The same was also observed at four weeks of age in both males (13.09 g ± 2.27 versus 13.45 g ± 2.11; n = 66 p = 0.516) and females (12.36 g ± 2.25 versus 12.39 g ± 1.78; n = 78 p = 0.945). Although data are presented separately for male and female animals, no significant difference in weight between wild type male and female animals was observed until 6 weeks of age.

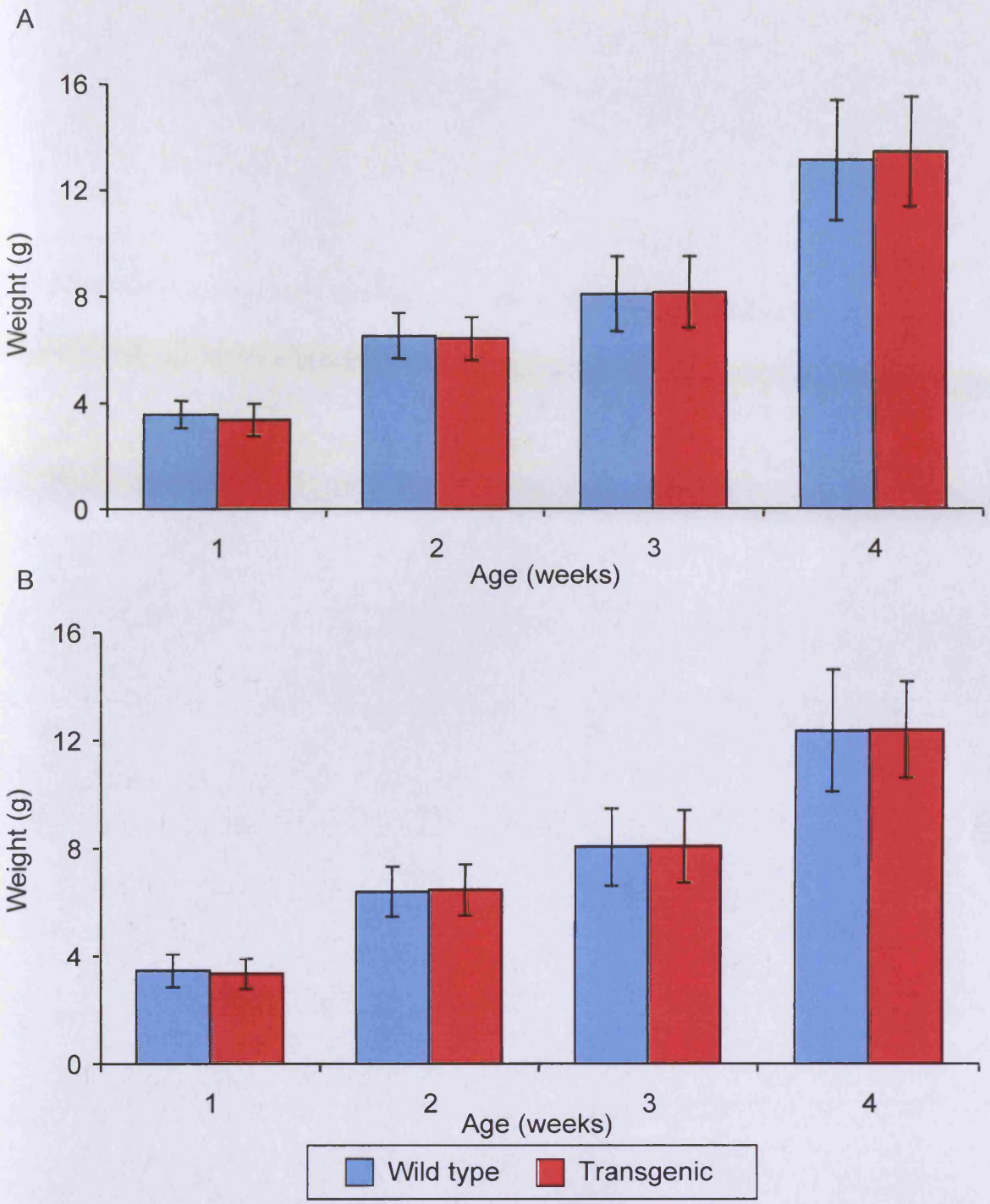


Figure 5.5: Weights of mice from line 10-15 prior to weaning

Mean raw weights of wild type and transgenic males (A) and females (B) from line 10-15 on a pure 129/Sv background from one week of age until weaning. Error bars denote standard deviation. Statistical significance was not achieved for any comparison examined by *t*-test.

5.2.3 Asymmetric growth restriction of transgenic mice

Rapid catch-up growth in the neo-natal period suggests that transgenic animals may be growth restricted due to placental insufficiency. Such growth phenotypes are characteristic of type II (asymmetric) IUGR, where the head and brain are proportionately larger in comparison to the growth-restricted body. Organ size was measured from P0 pups in order to determine whether growth restriction was symmetric or asymmetric.

Organ weights were measured in a subset of eight litters, which were born during the night over a five-day period. Entire litters were removed from the cage immediately prior to dissection and maintained at a warm temperature under a lamp. Transgenic animals within the cohort were 11.4% lighter than wild type (1.36 g ± 0.12 versus 1.20 g ± 0.12; n = 55; $p = 8.34 \times 10^{-6}$), and appeared to be asymmetrically growth restricted, with relatively large heads in comparison to body size. Organs were dissected, fixed in 4% PFA and equilibrated in 70% EtOH prior to weighing. Individual organ weights were normalised as described previously, and the mean weight for each organ in wild type and transgenic pups is presented in Figure 5.6. All organs were observed to be lighter in transgenic animals compared to wild type. The extent of this weight difference varied between approximately 5% and 20%. The brain of transgenic animals was 5.5% lighter than wild type (49.1 mg ± 2.68 versus 46.4 mg ± 3.00; n = 55; $p = 9.04 \times 10^{-4}$). Heart, liver and kidneys of transgenic animals were 12.1% (5.7 mg ± 0.57 versus 5.0 mg ± 0.94; n = 55; $p = 1.68 \times 10^{-3}$), 11.6% (41.1 mg ± 3.97 versus 36.7 mg ± 5.02; n = 55; $p = 7.74 \times 10^{-4}$) and 12.3% (4.1 mg ± 0.59 versus 3.6 mg ± 0.47; n = 110; $p = 2.76 \times 10^{-6}$) lighter than wild type respectively, exhibiting a similar degree of weight reduction compared to body weight. Lungs and pancreas of transgenic pups were 15.1% (32.2 mg ± 4.77 versus 27.3 mg ± 6.50; n = 55; $p = 2.49 \times 10^{-3}$) and 20.7% lighter than wild type respectively (8.7 mg ± 1.82 versus 6.9 mg ± 2.17; n = 55; $p = 1.57 \times 10^{-3}$).

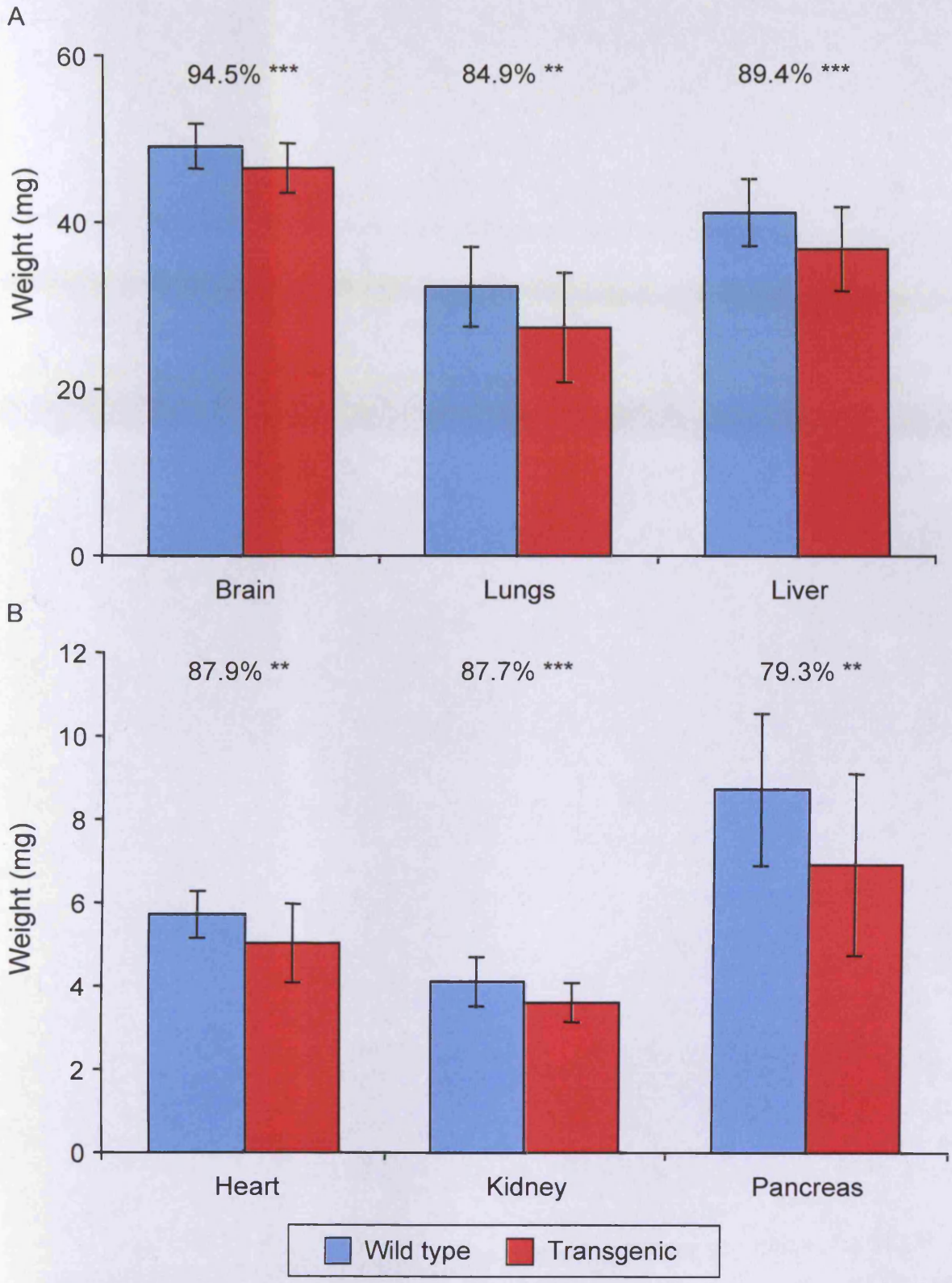


Figure 5.6: P0 organ weights from line 10-15

Neonatal pups of line 10-15 on a pure 129/Sv background were dissected and organ weights obtained post-fixation to determine the relative growth restriction of each organ. Percentage figures indicate the relative weight of transgenic organs compared to wild type. Error bars show standard deviation; asterisks denote significance level according to the Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

All organs examined displayed a significantly reduced weight in transgenic pups compared to wild type. However, by expressing organ weight as a proportion of body weight, the relative extent of growth restriction can be more directly observed. Data are presented in this format in Figure 5.7. Transgenic pups display a 6.6% increase in brain proportion compared to wild type, (3.65% ± 0.36 versus 3.89% ± 0.36; n = 55; p = 0.017). Heart, liver and kidneys exhibit a 0.9% decrease (0.42% ± 0.04 versus 0.42% ± 0.07; n = 55; p = 0.815), 0.6% increase (3.04% ± 0.25 versus 3.06% ± 0.30; n = 55; p = 0.799) and 0.5% decrease (0.30% ± 0.03 versus 0.30% ± 0.03; n = 110; p = 0.799) in body weight proportion respectively, without statistical significance. Lungs and pancreas were 4.2% lighter (2.37% ± 0.25 versus 2.27% ± 0.48; n = 55; p = 0.334) and 10.9% lighter (0.64% ± 0.11 versus 0.57% ± 0.16; n = 55; p = 0.071) as a proportion of body weight respectively, although both failed to achieve statistical significance.

We recently reported that transgenic embryos of line 5D3 display embryonic growth restriction from at least E13.5, with this weight deficit persisting into adulthood (Andrews *et al.* 2007). This transgenic line may potentially represent a model of symmetric IUGR, characterised by onset of IUGR during early gestation with an absence of post-natal catch up growth. Organ weights were also examined in neonatal animals from line 5D3 to examine if brain-sparing growth was present.

Brain sparing growth was not observed in transgenic neonates of line 5D3, with brain weight representing a comparable proportion of body weight in wild type and transgenic animals (3.00% ± 0.22 versus 2.92% ± 0.28; 6 litters; n = 38; p = 0.333) (Fig 5.8A). Similarly, no significant difference in body weight proportion was observed for lungs (2.47% ± 0.34 versus 2.66% ± 0.42; 6 litters; n = 38; p = 0.152), liver (3.13% ± 0.22 versus 3.13% ± 0.36; 6 litters; n = 38; p = 0.124), heart (0.43% ± 0.03 versus 0.45% ± 0.06; 6 litters; n = 38; p = 0.185), kidneys (0.29% ± 0.02 versus 0.30% ± 0.04; 6 litters; n = 76; p = 0.137) or pancreas (0.59% ± 0.06 versus 0.54% ± 0.13; 6 litters; n = 38; p = 0.124).

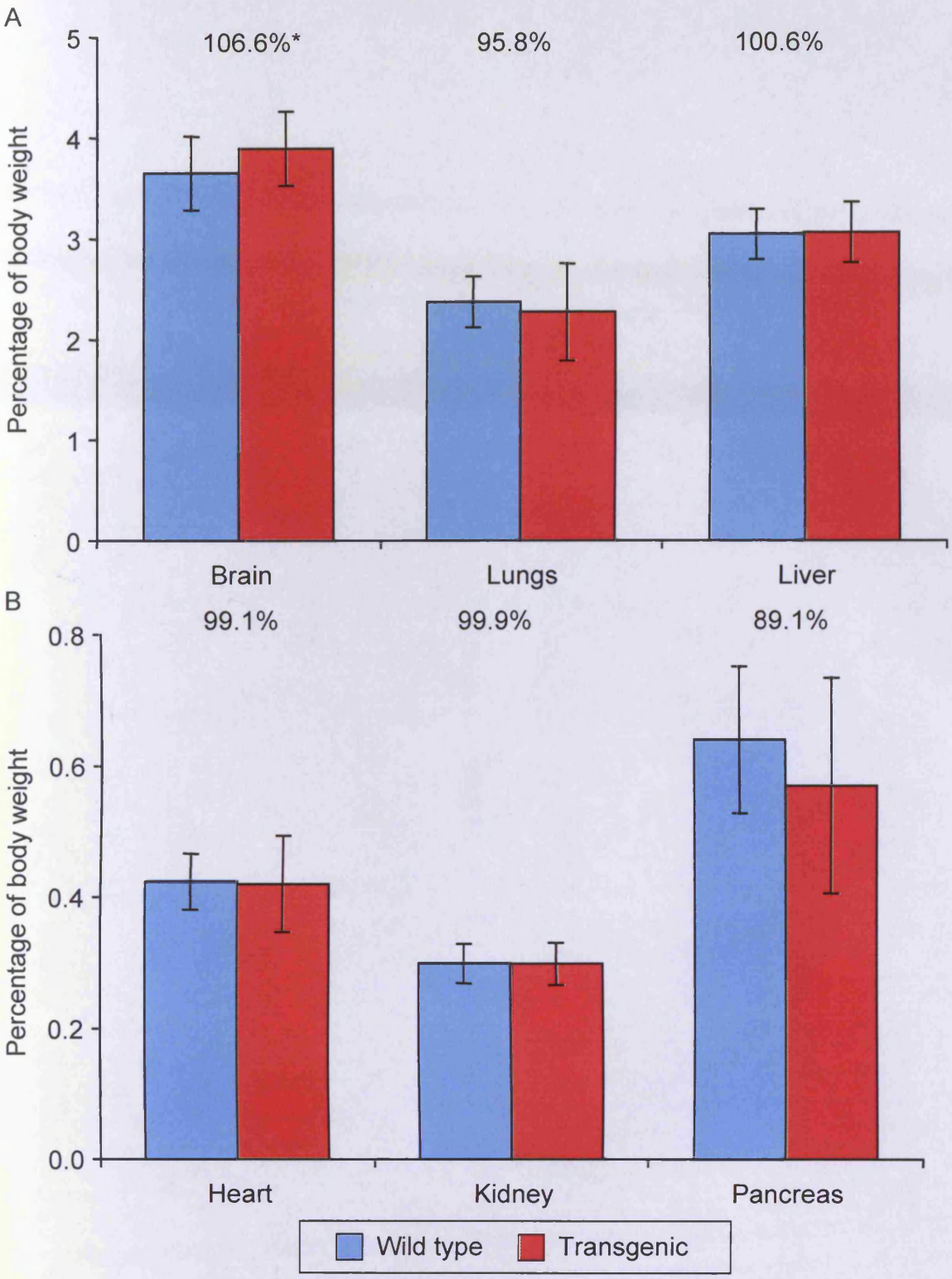


Figure 5.7: P0 organ weights relative to body weight from line 10-15

Organ weights were also examined as a proportion of body weight to assess the presence of brain-sparing growth. Normalised weights as a proportion of normalised body weight. Percentage figures indicate the relative proportion of transgenic organs compared to wild type. Error bars show standard deviation; asterisks denote significance level according to the *t*-test: * $p < 0.05$.

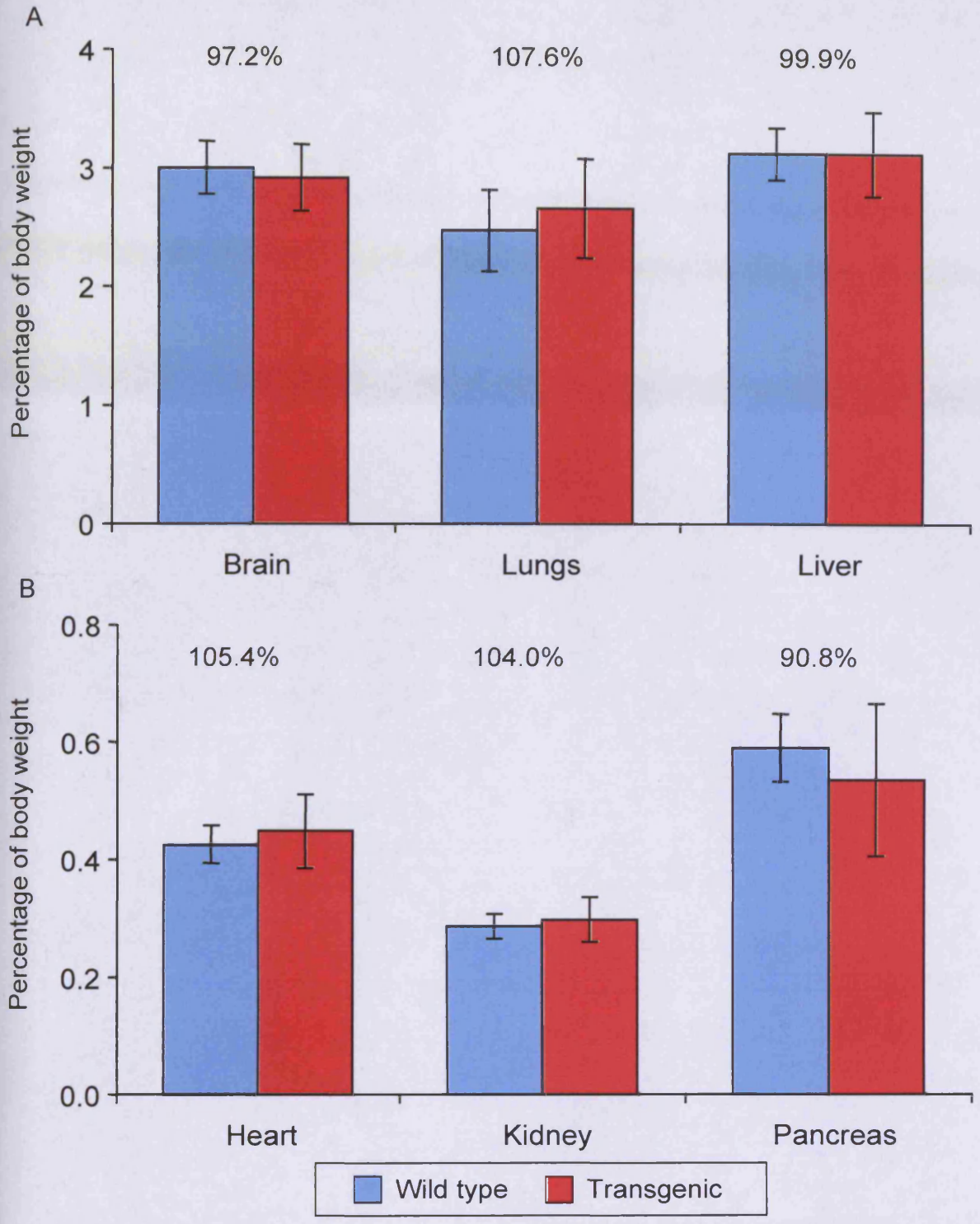


Figure 5.8: Organ weights relative to body weight from line 5D3

Organ weights were compared between wild type and transgenic neonates from line 5D3 on the 129/Sv x C57BL/6 background to determine if growth restriction was symmetric. Normalised weights as a proportion of normalised body weight. Percentage figures indicate the relative proportion of transgenic organs compared to wild type. Error bars show standard deviation.

5.2.4 Weight of adult transgenic animals is comparable to wild type

Asymmetric IUGR with catch-up growth has been linked with metabolic syndrome and obesity in humans (Eriksson *et al.* 1999; Forsen *et al.* 2000). A cohort of animals was weighed weekly from one week until ten weeks of age, and thereafter every fifth week until approximately one year of age. No significant difference in weight was observed between wild type and transgenic females at any age. Transgenic males were 16.2% larger than wild type males at 5 weeks (16.57 g ± 3.96 versus 19.26 g ± 3.05; n = 56; $p = 5.89 \times 10^{-3}$), 7.5% larger at 6 weeks (21.14 g ± 2.44 versus 22.72 g ± 1.84; n = 54; $p = 9.09 \times 10^{-3}$), 4.4% larger at 7 weeks (23.84 g ± 1.78 versus 24.89 g ± 1.65; n = 54; $p = 0.0295$) and 6.3% larger at 8 weeks of age (24.47 g ± 2.25 versus 26.00 g ± 1.82; n = 43; $p = 0.0189$). At 9 weeks of age, transgenic males were 3.5% larger than wild type, although this failed to achieve statistical significance (26.15 g ± 1.43 versus 27.06 g ± 1.80; n = 34; $p = 0.141$). There was subsequently no significant difference in weight observed between transgenic and wild type male mice between 10 weeks and one year of age. Growth of male and female animals between 5 and 50 weeks of age is shown in Figure 5.9.

Further to weighing a cohort of mice up to one year of age, a subset of animals were sacrificed at 10 weeks, 6 months and one year of age. Tibia bone lengths were measured from these animals to determine if transgenic and wild type animals achieved comparable growth rates. Average tibia lengths for male and female animals are presented in Figure 5.10. At all ages examined, female transgenic mice were comparable to wild type, with no significant difference in tibia length at 10 weeks (1.77 cm ± 0.060 versus 1.76 ± 0.049; n = 6; $p = 0.513$), 6 months (1.84 cm ± 0.045 versus 1.85 ± 0.036; n = 8; $p = 0.773$) or 1 year (1.83 cm ± 0.036 versus 1.83 ± 0.036; n = 12; $p = 0.570$) of age. Similarly, male transgenic animals showed no difference in tibia length at 10 weeks (1.78 cm ± 0.030 versus 1.80 ± 0.038; n = 6; $p = 0.275$) or 6 months (1.83 cm ± 0.023 versus 1.80 ± 0.031; n = 8; $p = 0.134$) of age, although at one year of age, transgenic male mice had slightly shorter tibia compared to wild type (1.89 cm ± 0.032 versus 1.84 ± 0.032; n = 12; $p = 0.028$).

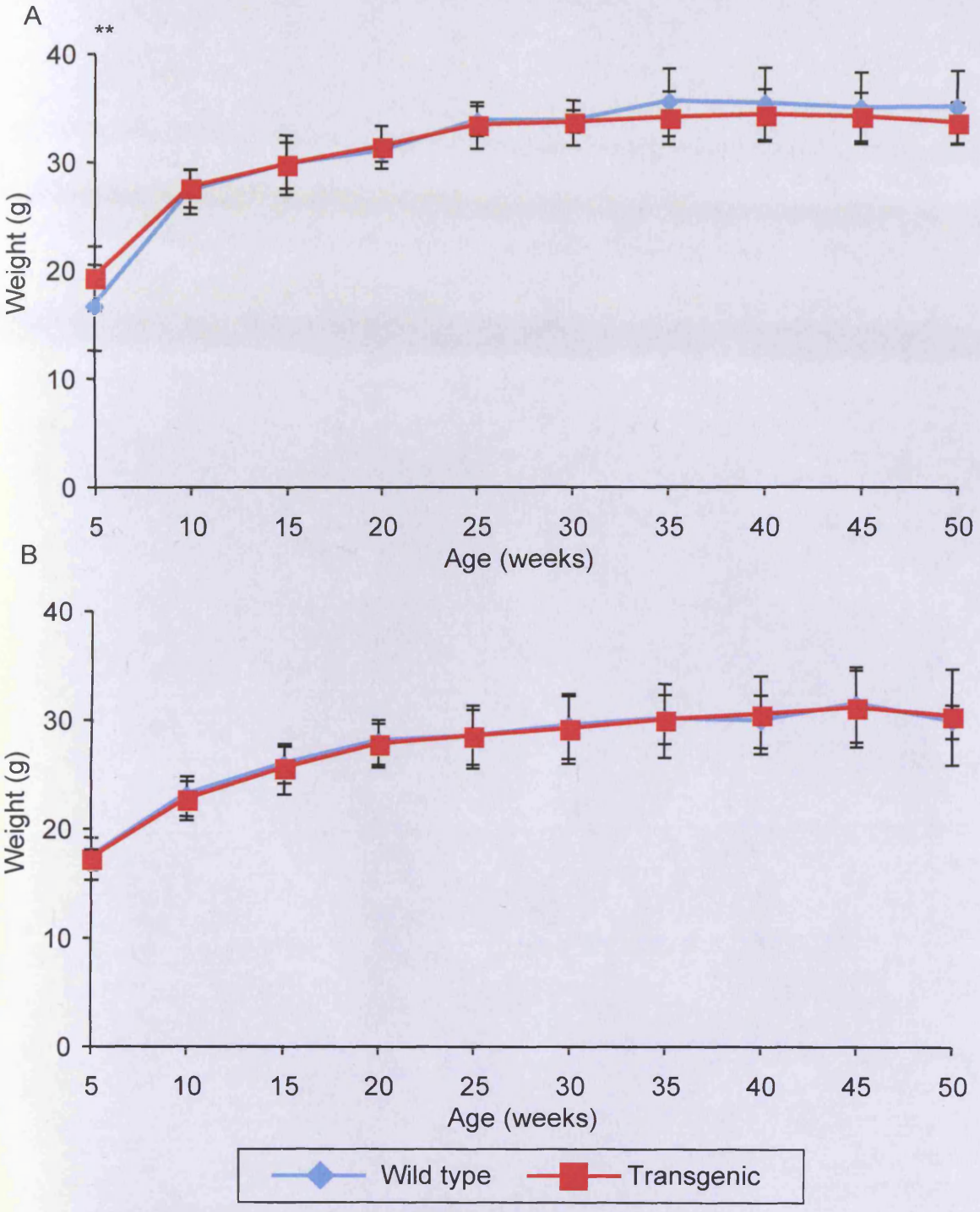


Figure 5.9: Adult weights of male and female mice from line 10-15

Adult mice from line 10-15 on a pure 129/Sv background were weighed every fifth week over the course of a year to assess weight gain of wild type and transgenic animals. Mean raw weights are presented separately for A) males and B) females. Error bars show standard deviation; asterisks denote significance level according to the Mann-Whitney test: ** $p < 0.01$.

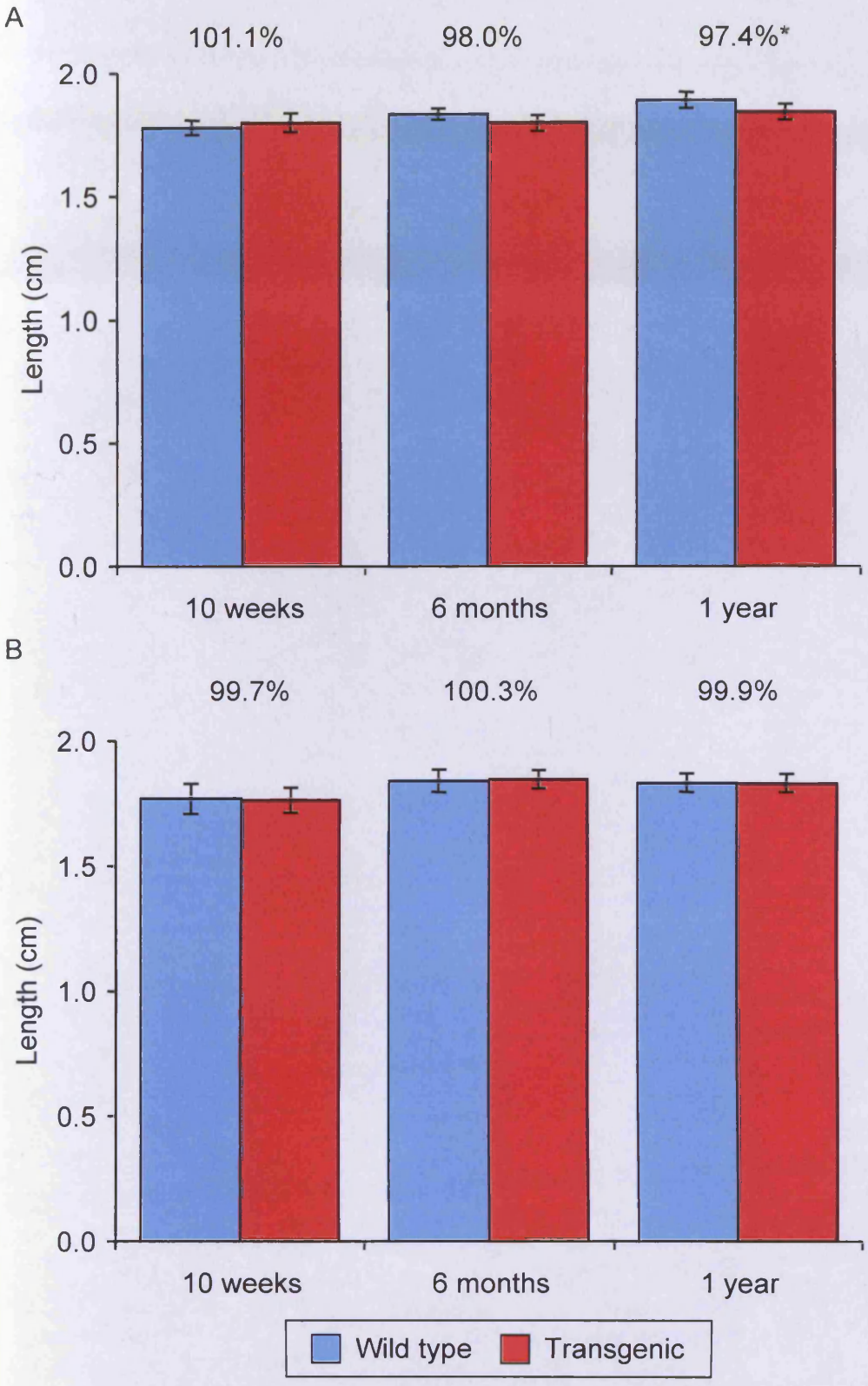


Figure 5.10: Tibia length of adult animals from line 10-15

Tibia length of adult mice from line 10-15 on a pure 129/Sv background was determined at the ages shown to examine if wild type and transgenic animals are proportionate in length. Mean raw tibia lengths are shown for A) male and B) female wild type and transgenic mice at 10 weeks, 6 months and 1 year of age. Error bars show standard deviation; asterisks denote significance level according to the Mann-Whitney test: * $p < 0.05$.

5.2.5 Fat deposition is increased in female transgenic mice

No overt sustained difference in weight was observed in transgenic mice of either sex compared to wild type animals over the course of one year. To specifically examine adipose mass, the retroperitoneal and mesenteric fat depots were dissected from mice sacrificed at 10 weeks, 6 months and 1 year of age. These fat deposits were weighed and morphology examined by H&E staining. Mean weights of the individual fat depots and the combined weight of the two fat depots are presented in Figures 5.11 (females) and 5.12 (males).

No significant weight difference was observed between wild type and transgenic mesenteric (402.2 mg ± 66.6 versus 370.6 mg ± 61.2; n = 14; p = 0.338) or retroperitoneal (68.3 mg ± 19.7 versus 69.4 mg ± 18.3; n = 14; p = 0.898) fat depots in females at 10 weeks of age. The mesenteric fat of transgenic females was slightly lighter than wild type, resulting in a ~7.5% reduction in combined fat weight (470.5 mg ± 76.3 versus 440.0 mg ± 58.7; n = 14; p = 0.406), although this did not reach significance. At 6 months, there was no difference in weight of mesenteric fat depots (324.3 mg ± 25.7 versus 321.8 mg ± 44.8; n = 8; p = 0.773), and a ~30% increase in the weight of retroperitoneal adipose tissue (69.0 mg ± 9.4 versus 90.3 mg ± 18.9; n = 8; p = 0.083), although this failed to reach statistical significance. Due to the relatively heavier mesenteric fat exhibiting no increase in mean weight, the combined fat weight was no different in transgenic females (393.3 mg ± 33.6 versus 412.0 mg ± 56.3; n = 8; p = 0.564). At one year of age, the mean weight of mesenteric fat of females was ~70% greater (228.3 mg ± 64.8 versus 382.3 mg ± 242.4; n = 12; p = 0.223), with a ~60% increase in the weight of retroperitoneal fat (96.0 mg ± 12.2 versus 150.3 mg ± 93.5; n = 12; p = 0.062), although neither result achieved statistical significance. The weight of the combined fat pads achieved marginal statistical significance, with a ~65% increase in weight of transgenic adipose tissue compared to wild type (324.3 mg ± 60.2 versus 532.6 mg ± 332.0; n = 12; p = 0.042). The standard deviation of individual and combined fat pad weights in transgenic females at one year of age represented up to ~70% of the mean weight, whereas all other standard deviations were a maximum of 25% of the mean weight.

A similar pattern of weight gain was observed in transgenic male animals between 10 weeks and 6 months, although at 1 year of age transgenic fat pads were lighter than wild type, generally due to a large variation of wild type fat pad weights at this age. At 10 weeks of age, there was no significant difference in mesenteric fat (354.0 mg ± 104.4 versus 355.4 mg ± 94.2; n = 14; p = 0.468), with a ~14% reduction in retroperitoneal weight (72.9 mg ± 17.7 versus 63.0 mg ± 18.2; n = 14; p = 0.366), although this failed to reach statistical significance. There was no difference in combined fat pad weight (426.9 mg ± 106.2 versus 418.3 mg ± 85.9; n = 14; p = 0.897). At six months, transgenic animals exhibited a 17% increase in weight of both mesenteric (216.0 mg ± 46.6 versus 251.8 mg ± 54.6; n = 8; p = 0.881) and retroperitoneal (76.0 mg ± 37.3 versus 89.0 mg ± 31.6; n = 8; p = 0.655) fat pads, with a similar increase observed when these results were combined (292.0 mg ± 78.6 versus 340.8 mg ± 81.4; n = 8; p = 0.881), although again statistical significance was not achieved. At one year of age there was a ~25% reduction in mesenteric (368.1 mg ± 154.1 versus 278.4 mg ± 70.9; n = 12; p = 0.416) and retroperitoneal (174.9 mg ± 118.7 versus 134.6 mg ± 78.2; n = 12; p = 0.685) adipose depots of transgenic animals. A similar difference was observed when the combined data was analysed (543.0 mg ± 265.7 versus 413.0 mg ± 108.3; n = 12; p = 0.570). In contrast to females, at one year of age the male animals displayed greater variability in individual fat pad weights.

A comparison of raw fat pad weights may be complicated by variations in body weight between individual animals, and thus fat pad weights were also examined as a proportion of body weight. The mean results of this comparison are presented in Figures 5.13 (females) and 5.14 (males).

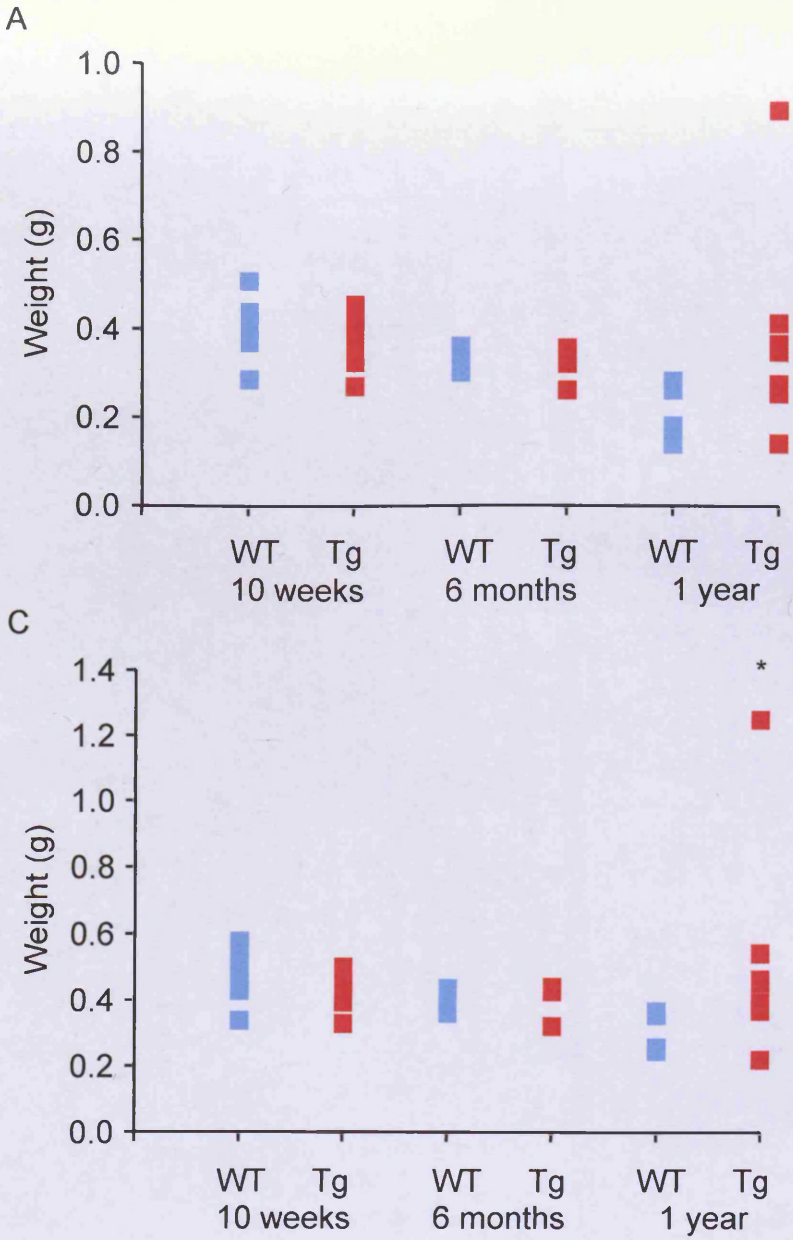


Figure 5.11: Fat pad weight of female mice from line 10-15

Weight of A) mesenteric, B) retroperitoneal and C) combined mesenteric and retroperitoneal fat from male mice at 10 weeks, 6 months and 1 year from line 10-15 on a pure 129/Sv background. Statistical significance calculated by the Mann-Whitney test is denoted by asterisks: * $p < 0.05$.

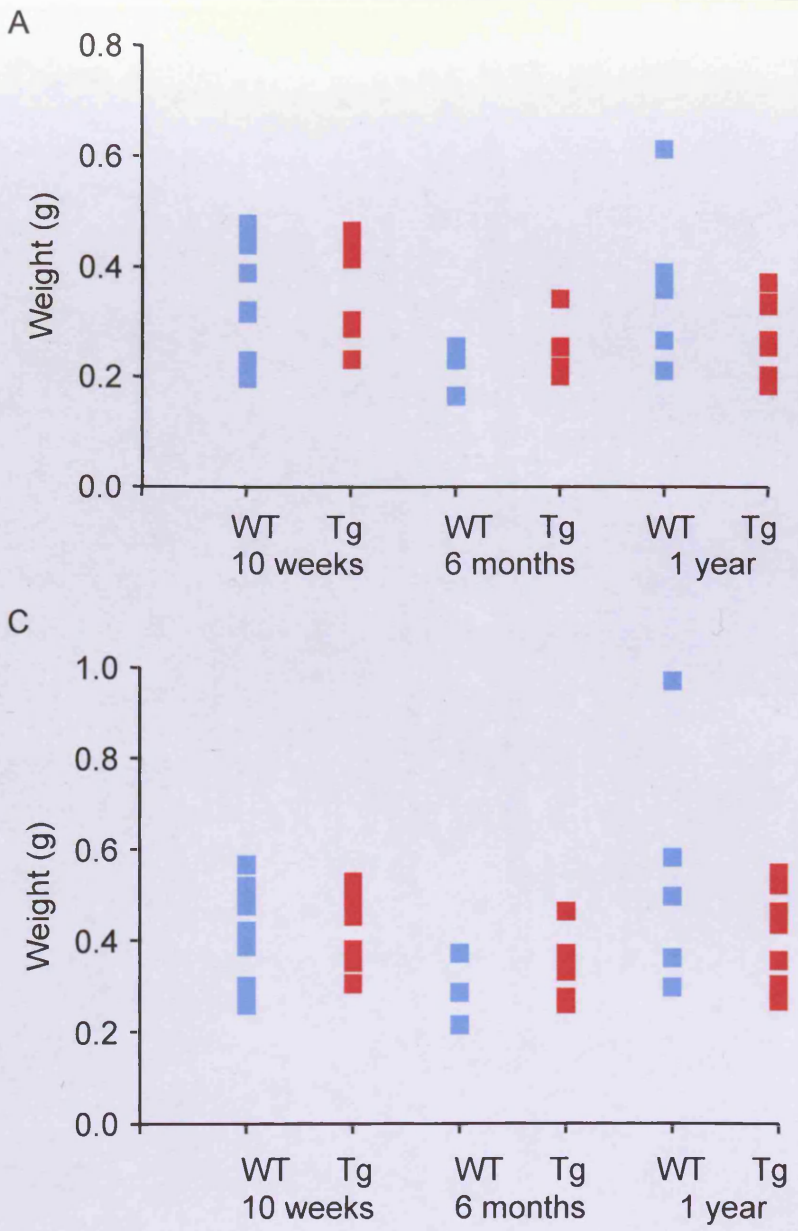


Figure 5.12: Fat pad weight of male mice from line 10-15

Weight of A) mesenteric, B) retroperitoneal and C) combined mesenteric and retroperitoneal fat from male mice at 10 weeks, 6 months and 1 year from line 10-15 on a pure 129/Sv background.

At 10 weeks of age, there was no significant difference in proportion of mesenteric (1.77% ± 0.29 versus 1.65% ± 0.31; n = 14; p = 0.406) or retroperitoneal (0.30% ± 0.07 versus 0.30% ± 0.06; n = 14; p = 0.949) fat in female animals, consistent with the combined data (2.07% ± 0.31 versus 1.95% ± 0.28; n = 14; p = 0.338). At 6 months of age there was no difference in the proportion of mesenteric fat pad (1.15% ± 0.10 versus 1.18% ± 0.23; n = 8; p = 0.773), with a ~34% increase in the proportion of retroperitoneal fat (0.24% ± 0.04 versus 0.33% ± 0.06; n = 8; p = 0.083), although this did not achieve statistical significance. No difference was observed in fat pad proportion of the combined fat pad weight (1.39% ± 0.14 versus 1.51% ± 0.26; n = 8; p = 0.386). At one year of age, mesenteric fat pad proportion was ~60% greater in transgenics (0.77% ± 0.21 versus 1.21% ± 0.57; n = 8; p = 0.062), with a ~50% increase observed for retroperitoneal fat (0.32% ± 0.03 versus 0.47% ± 0.21; n = 8; p = 0.062). The combined proportion of mesenteric and retroperitoneal fat in transgenic females was ~55% more (1.09% ± 0.18 versus 1.68% ± 0.76; n = 8; p = 0.042). Normalisation of fat pad weight according to individual body weight did not affect the standard deviation of transgenic fat pad proportions at one year of age, suggesting that fat pad weight was not necessarily correlated with body weight.

Male animals at 10 weeks of age did not exhibit any difference in fat pad proportion of mesenteric (1.35% ± 0.38 versus 1.38% ± 0.30; n = 14; p = 0.698), retroperitoneal (0.28% ± 0.07 versus 0.25% ± 0.70; n = 14; p = 0.271), or combined (1.63% ± 0.39 versus 1.63% ± 0.25; n = 14; p = 0.796). At six months of age, fat pad proportions of mesenteric (0.75% ± 0.16 versus 0.84% ± 0.16; n = 8; p = 0.881), retroperitoneal (0.26% ± 0.13 versus 0.30% ± 0.10; n = 8; p = 0.881) and combined (1.01% ± 0.27 versus 1.14% ± 0.25; n = 8; p = 0.655) fat pads were increased by approximately 12%. At one year of age, fat pad proportions were reduced by approximately 20% for mesenteric (1.06% ± 0.37 versus 0.83% ± 0.23; n = 12; p = 0.570), retroperitoneal (0.49% ± 0.28 versus 0.39% ± 0.20; n = 12; p = 0.465) and combined (1.55% ± 0.61 versus 1.22% ± 0.30; n = 12; p = 0.291) fat pads, although statistical significance was not achieved in any instance. Interestingly, the large standard deviation associated with fat pad weight in wild type animals was reduced when corrected for body weight, suggesting that male fat pad weight is directly correlated with body mass.

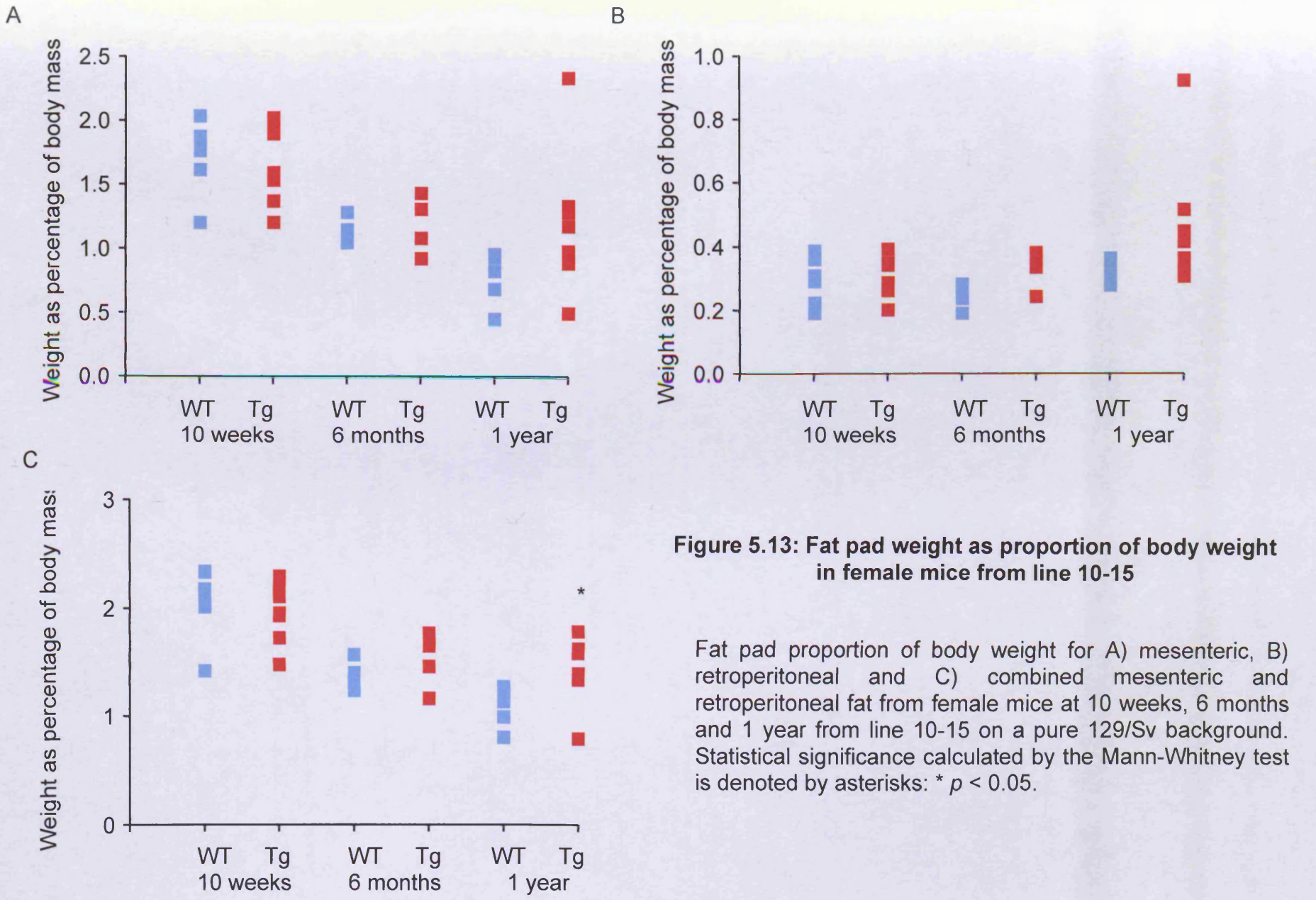


Figure 5.13: Fat pad weight as proportion of body weight in female mice from line 10-15

Fat pad proportion of body weight for A) mesenteric, B) retroperitoneal and C) combined mesenteric and retroperitoneal fat from female mice at 10 weeks, 6 months and 1 year from line 10-15 on a pure 129/Sv background. Statistical significance calculated by the Mann-Whitney test is denoted by asterisks: * $p < 0.05$.

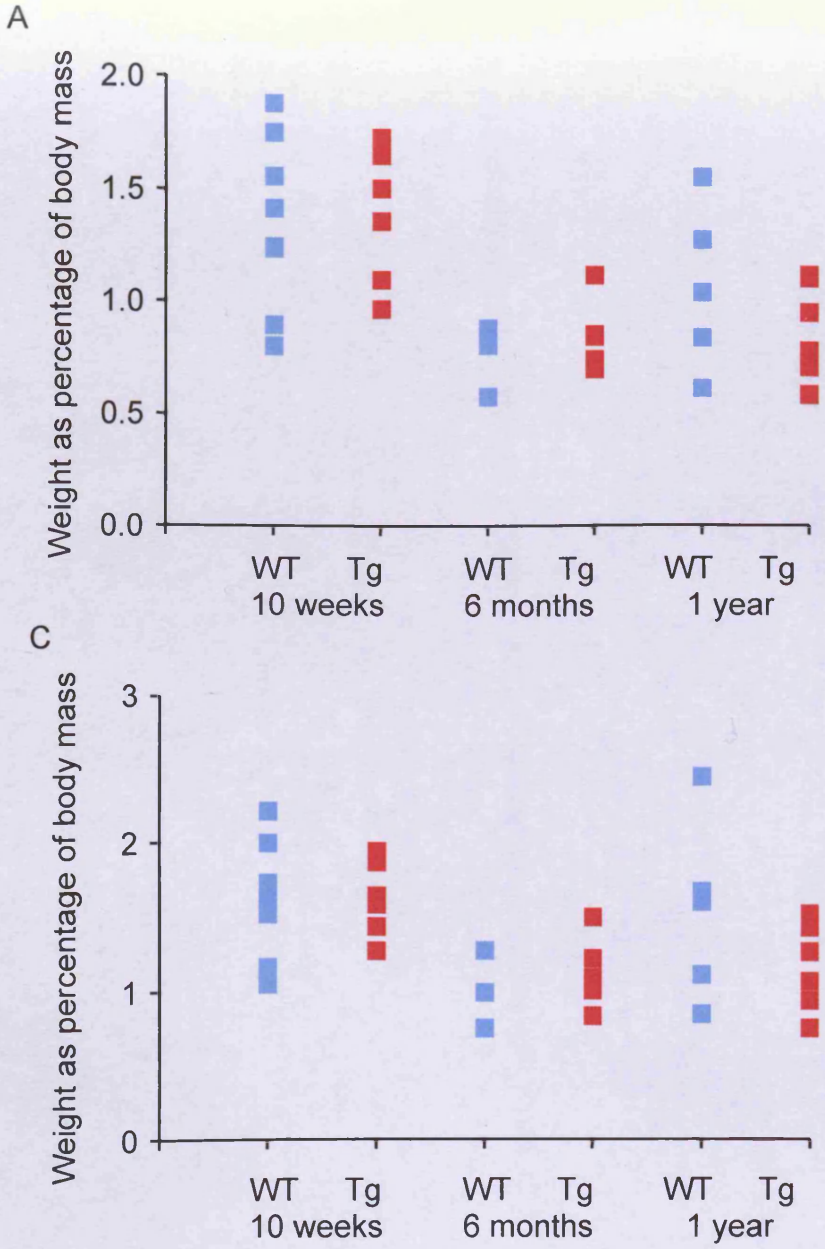


Figure 5.14: Fat pad weight as proportion of body weight in male mice from line 10-15

Fat pad proportion of body weight for A) mesenteric, B) retroperitoneal and C) combined mesenteric and retroperitoneal fat from female mice at 10 weeks, 6 months and 1 year from line 10-15 on a pure 129/Sv background.

The modest increase in fat pad weight observed in transgenic females at one year of age may result from an increase in either cell size or cell number, or a combination of both. Adipocyte cell diameter was measured according to the method of Ashwell and colleagues (1976), which was modified as described in Section 2.15.2. Mean cell diameter was calculated from the number of cells counted in a defined region. Three wild type and three transgenic animals were examined at 10 weeks and 1 year of age, with three regions of retroperitoneal fat measured from each individual. H&E stained sections representative of each genotype at both stages are shown in Figure 5.15 A-D. The mean cell diameter at each stage is shown in Figure 5.15 E, with error bars representing the standard deviation. No significant difference was observed in adipocyte size between wild type and transgenic animals at 10 weeks (52.1 μm ± 3.0 versus 53.1 μm ± 2.8; n = 6; p = 0.773) or one year of age (67.9 μm ± 2.3 versus 65.6 μm ± 1.9; n = 6; p = 0.439).

The hormone leptin is secreted by adipose tissue, with circulating serum leptin concentration tending to correlate with the degree of adiposity. Plasma leptin concentration of female animals at 6 months and one year of age was determined by ELISA. Blood was taken at the terminal end-point of the same animals from which fat pads were dissected. Mice were not fasted prior to dissection, and thus the plasma concentration represents mice in a fed state. Individual serum leptin concentrations are presented in Figure 5.16 A. At 6 months of age, there was no difference in serum leptin concentration between wild type and transgenic animals (80.8 ρM ± 42.1 versus 87.0 ρM ± 33.1; n = 8; p = 0.773), whereas at one year of age, despite a large degree of variation, transgenic females exhibited a significantly elevated plasma leptin concentration (43.9 ρM ± 28.1 versus 236.6 ρM ± 277.7; n = 11; p = 0.028). Leptin concentration was also normalised according to the combined fat pad weight of the individual and analysed separately (Fig. 5.16 B). A similar pattern of results was observed, with no discernable difference between wild type and transgenic animals at 6 months (0.207 ρM/mg ± 0.117 versus 0.212 ρM/mg ± 0.073; n = 8; p = 0.564), and a marginally significant difference at 1 year of age (0.152 ρM/mg ± 0.124 versus 0.323 ρM/mg ± 0.164; n = 11; p = 0.045).

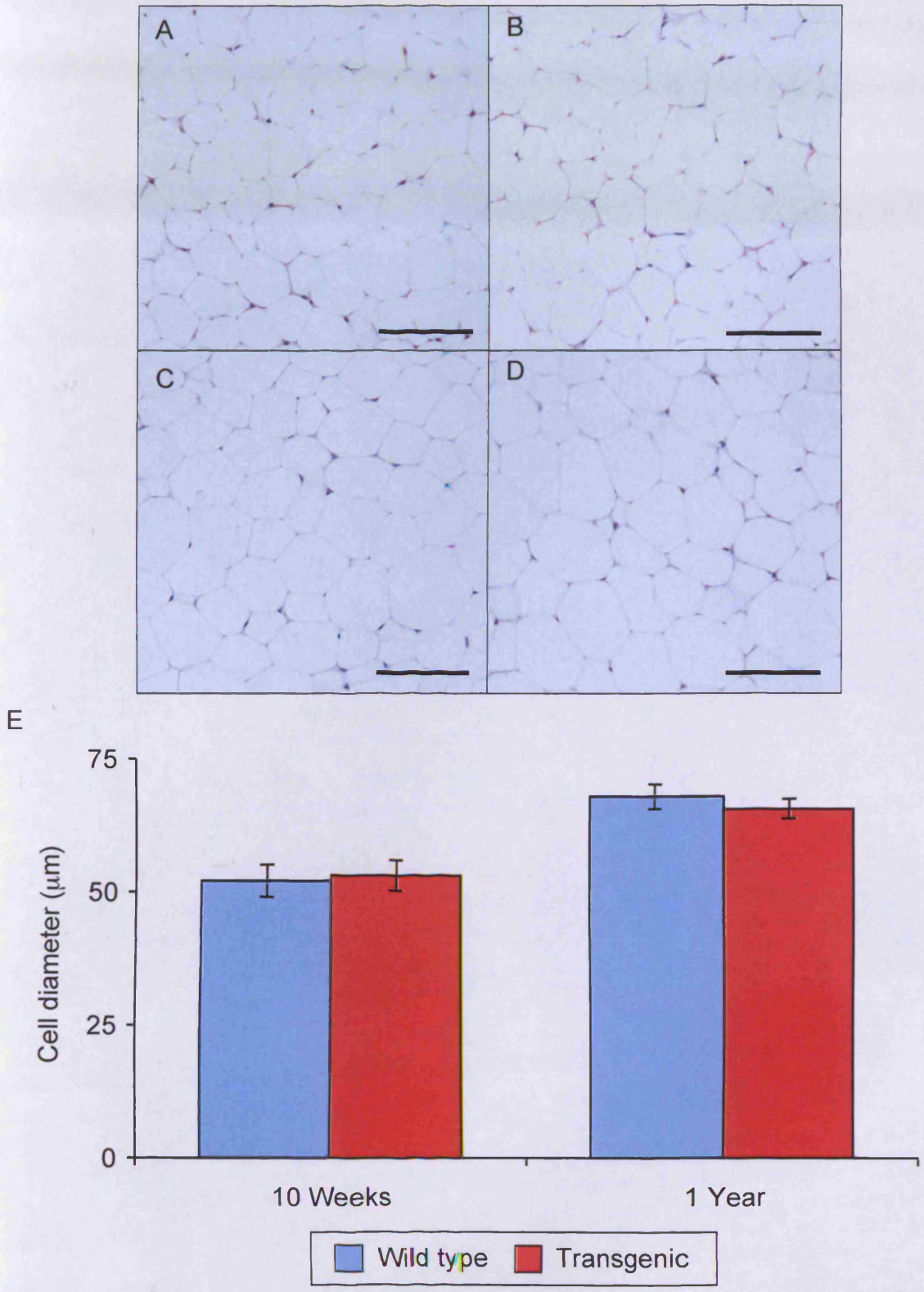


Figure 5.15: Morphological analysis of retroperitoneal adipose tissue

H&E stained retroperitoneal adipose depots of wild type and transgenic females from line 10-15 on a pure 129/Sv background at 10 weeks (A and B respectively) and 1 year (C and D respectively) of age. Scale bar = 100 μm. Graph in E shows the average adipose cell diameter. Error bars represent standard deviation.

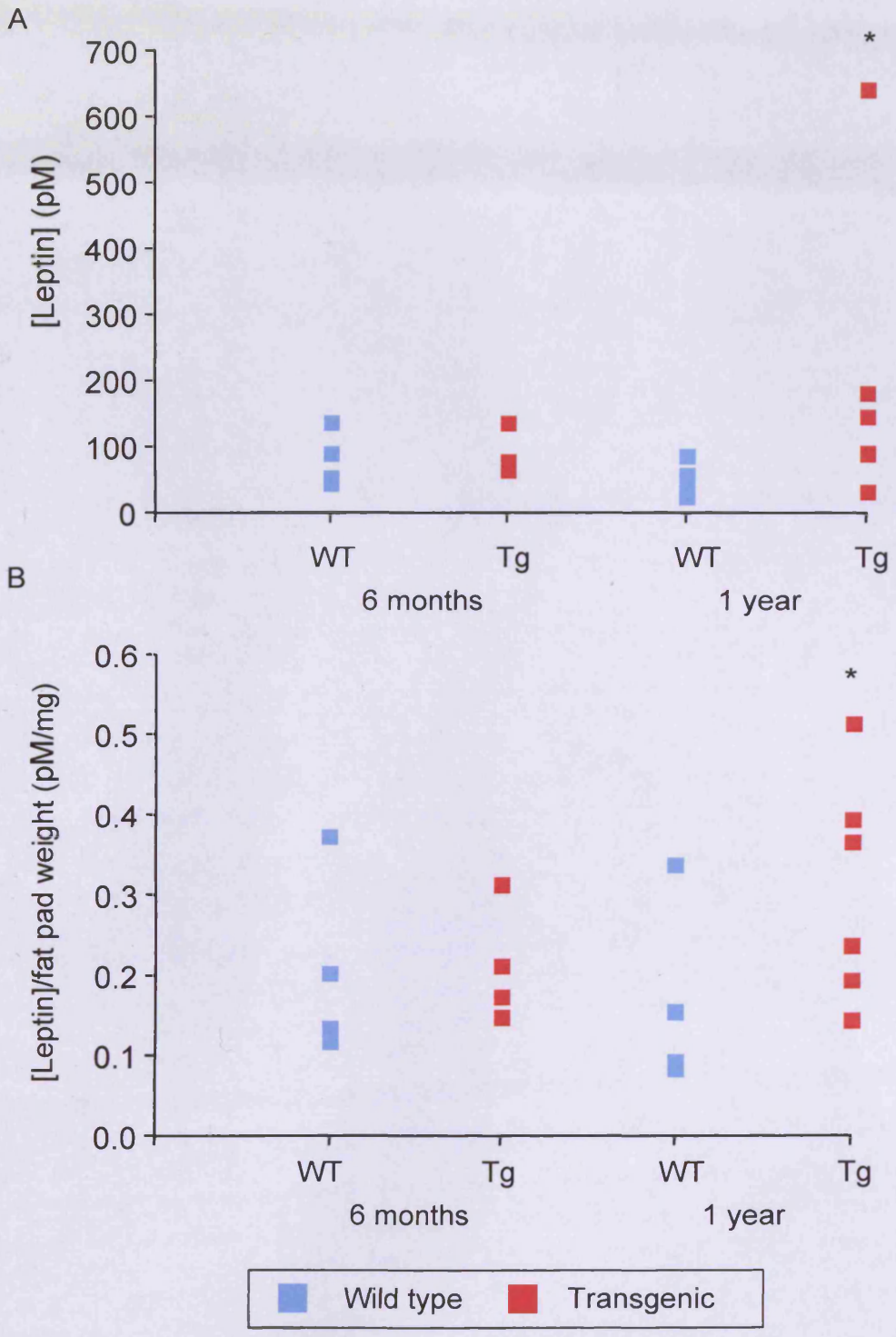


Figure 5.16: Serum leptin concentration in females

Scatter plots showing A) the serum leptin concentration in females at six months and one year of age; and B) the serum leptin concentration normalised to fat pad weight at the same stages in animals from line 10-15 on a pure 129/Sv background. Significance levels as calculated by the Mann Whitney test are denoted by asterisks; * $p < 0.05$.

One possible explanation for the increased adiposity observed in transgenic animals is that *Phlda2* and/or *Slc22a18* are over-expressed in adipose tissue. Semi-quantitative PCR was initially used to determine if either gene was endogenously expressed in wild type adipose tissue or if transcripts could be detected in adipose tissue of transgenic animals. No transcript could be detected for *Phlda2*, with relatively low levels of *Slc22a18* expression in white adipose tissue. *Cdkn1c* was also expressed in both wild type and transgenic adipose tissue (Fig 5.17). Transcripts for *Slc22a18* and *Phlda2* could not be detected by qPCR, despite the amplification of both reference gene transcripts with C_T values of between 18 and 20, indicating that *Phlda2* is not expressed in white adipose tissue and that *Slc22a18* expression is minimal.

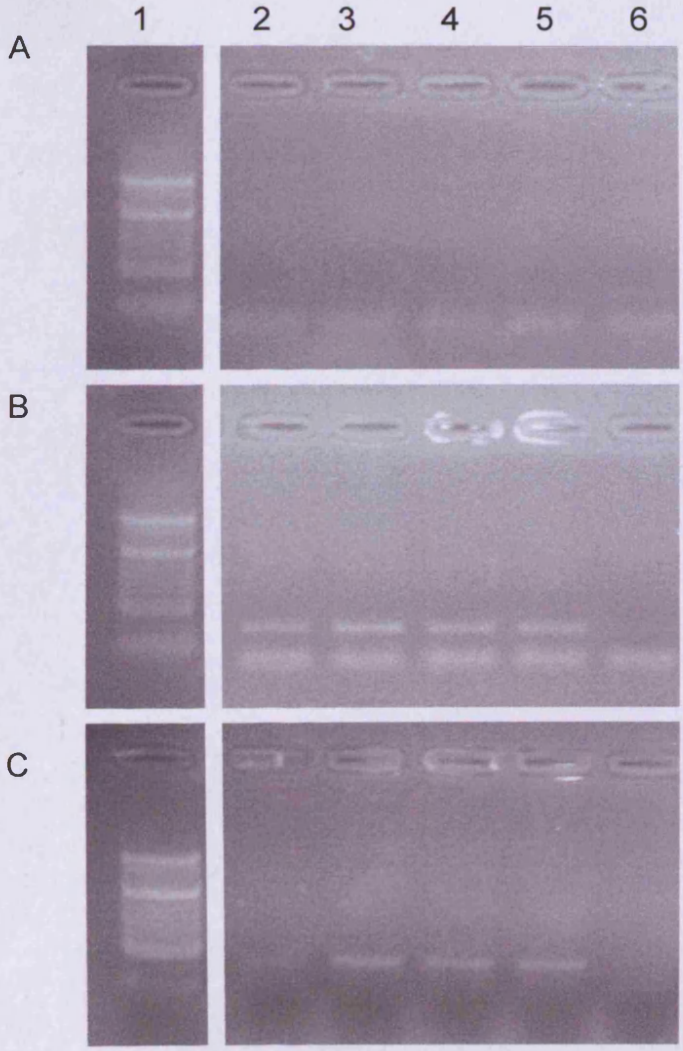


Figure 5.17: Analysis of gene expression in retroperitoneal adipose tissue

Expression of A) *Phlda2*, B) *Cdkn1c* and C) *Slc22a18* in adult retroperitoneal adipose depots of wild type (lanes 2 and 3) and transgenic animals (lanes 4 and 5) from line 10-15 on a pure 129/Sv background. Lane 1 shows a 100 bp ladder and lane 6 is a negative (water) control.

There are several of disadvantages of assessing adiposity from dissecting the tissue from animals and weighing it. Primarily this approach requires a larger cohort of animals than would be required if fat deposition could be measured without needing to sacrifice the animals. Additionally, the approach is limited by the accuracy of dissection and access to various sites of adipose tissue. An alternative method to dissecting fat pads at various stages is to use Magnetic Resonance Imaging (MRI) to directly quantify adipose tissue in live animals. In order to establish this technique for use in future experiments, a small cohort of animals was subjected to MRI scanning. Due to restrictions of our current Home Office licensing, animals used in this study were sacrificed immediately prior to scanning. However, future use of this technique would enable the use of anaesthetised animals such that fat deposition could be investigated in a longitudinal study utilising the same cohort of animals, in which glucose and insulin tolerance testing would also be performed.

A cohort of fourteen females at 14 months of age, consisting of four transgenics and 10 wild type were selected for this study, with total body fat volume calculated from MRI scan images as described in Section 2.17, and combined adipose to body weight proportions calculated from dissection of fat pads immediately following MRI scan. As observed previously, no significant difference was observed in body weight of transgenic mice compared to wild type (31.1 g ± 3.2 versus 31.1 mg ± 1.9; n = 14; p = 0.733). As a proportion of body weight, the combined fat pad weight was not significantly different in transgenic animals compared to wild type (1.69% ± 0.54 versus 1.76% ± 0.12; n = 14; p = 0.540). Similarly, following analysis of MRI scan images, fat volume as a proportion of total body volume was also not significantly different in transgenic animals compared to wild type (20.6% ± 4.2 versus 22.2% ± 6.39; n = 14; p = 0.945). The relative volume of adipose tissue determine by MRI scanning was greater than the proportion indicated by dissection as the MRI enables analysis of total body fat, whereas only two adipose depots were examined by dissection.

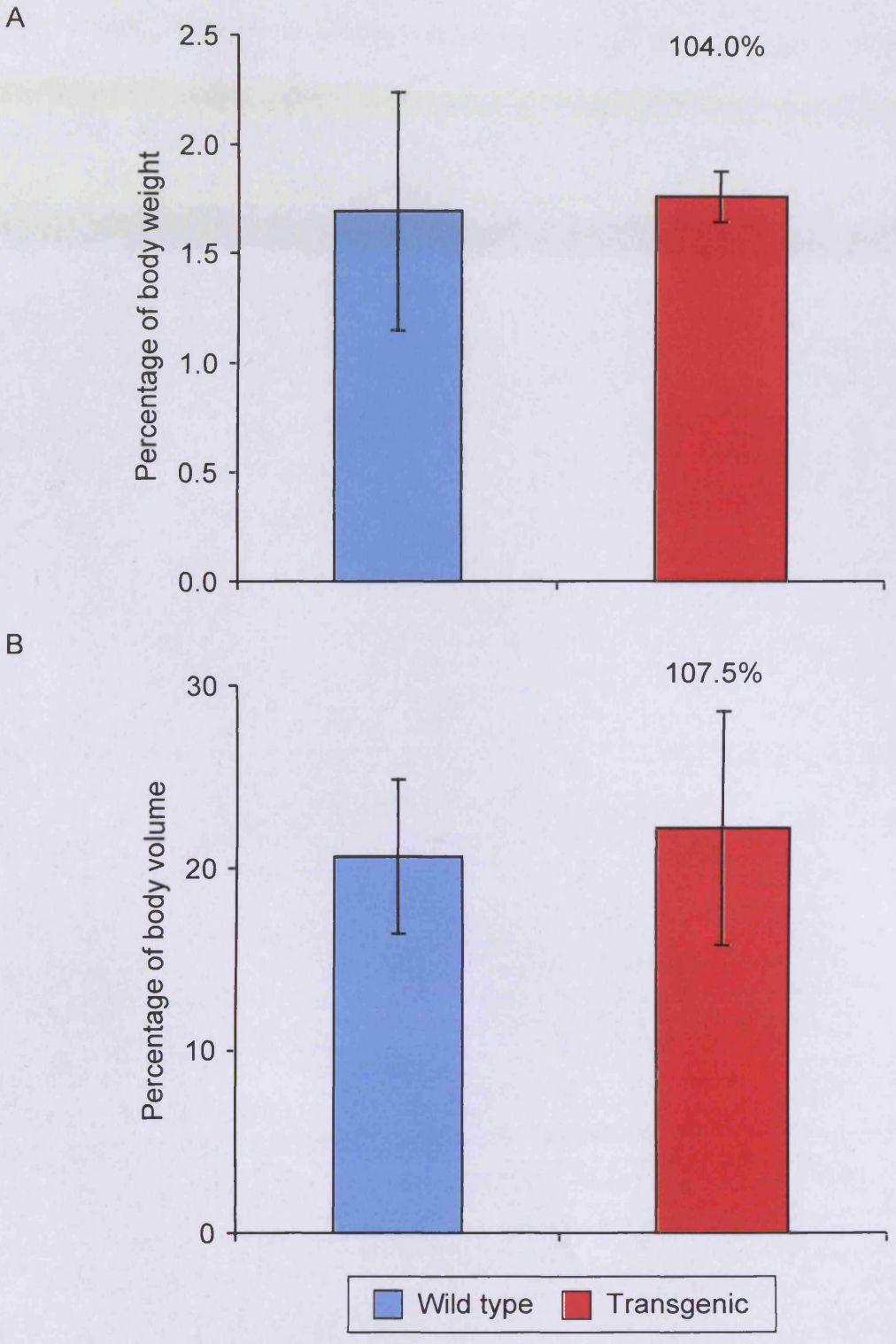


Figure 5.18: Comparison of adiposity determined by weight and MRI scans

Analysis of adiposity by MRI scans was compared with weights obtained by dissection from a cohort of 14 female mice from line 10-15 on a pure 129/Sv background. A) Combined retroperitoneal and mesenteric adipose tissue weights as a proportion of body weight and B) Total adipose tissue volume as a proportion of body volume

5.2.6 Female transgenic mice develop glucose intolerance

Increased visceral adiposity is a major risk factor for type 2 diabetes. Fasting glucose tolerance tests were performed on the same cohort of male and female mice at 10 weeks, 6 months and 1 year of age. Glucose tolerance tests were performed as described in Section 2.16. Mice were fasted overnight, in order to eliminate the possibility that recently consumed food would interfere with the test, and a baseline blood glucose measurement obtained. Each mouse was then injected with 2 mg/g glucose and blood glucose measured every thirty minutes thereafter. A normal insulin response would result in an initial peak blood-glucose concentration immediately after injection, followed by a steady decline in blood glucose as the metabolism responds to insulin. Glucose intolerance, or insulin insensitivity, was indicated by a delayed response to glucose injection, with blood glucose concentration remaining elevated in comparison to wild type animals of the same age.

Fasting blood glucose levels were no different in transgenic females at 10 weeks (3.46 mM ± 0.40 versus 3.64 mM ± 0.46; n = 12; p = 0.626) or 6 months (4.30 mM ± 1.06 versus 3.74 mM ± 0.54; n = 12; p = 0.086) of age. At one year of age, transgenic females exhibited slightly reduced fasting blood glucose levels (4.46 mM ± 0.65 versus 3.70 mM ± 0.43; n = 12; p = 0.051), although this failed to achieve statistical significance. The response to glucose challenge at 10 weeks was comparable between wild type and transgenic females, with no difference in blood glucose concentration 30 minutes (11.52 mM ± 1.01 versus 10.99 mM ± 1.23; n = 12; p = 0.370), 60 minutes (8.20 mM ± 0.88 versus 8.46 mM ± 1.44; n = 12; p = 0.870), 90 minutes (7.00 mM ± 0.76 versus 7.16 mM ± 0.50; n = 12; p = 0.515) or 120 minutes (6.06 mM ± 0.52 versus 6.41 mM ± 0.40; n = 12; p = 0.740) following glucose injection. A similar scenario was observed at 6 months of age, with no alteration to blood glucose concentration 30 minutes (9.28 mM ± 1.10 versus 9.56 mM ± 1.24; n = 12; p = 0.807), 60 minutes (7.38 mM ± 0.58 versus 7.84 mM ± 0.88; n = 12; p = 0.166), 90 minutes (6.52 mM ± 0.68 versus 6.39 mM ± 0.76; n = 12; p = 0.988) or 120 minutes (6.10 mM ± 0.46 versus 5.63 mM ± 0.92; n = 12; p = 0.566) following injection. In contrast, at one year of age, transgenic females displayed significantly elevated blood glucose concentration 30 minutes (7.88 mM ± 0.50 versus 8.89 mM ± 1.13; n = 12; p =

0.042) and 60 minutes (6.68 mM ± 0.59 versus 8.17 mM ± 2.03; n = 12; p = 0.041) following injection. Blood glucose concentration remained elevated 90 minutes (5.96 mM ± 1.05 versus 7.13 mM ± 1.14; n = 12; p = 0.057) and 120 minutes (5.50 mM ± 1.15 versus 6.36 mM ± 1.22; n = 12; p = 0.416) after injection, although without reaching statistical significance.

Fasting blood glucose levels were no different in transgenic males at 10 weeks (3.96 mM ± 0.92 versus 3.07 mM ± 0.80; n = 12; p = 0.106), 6 months (3.98 mM ± 0.75 versus 3.29 mM ± 0.25; n = 12; p = 0.106) or 1 year (3.86 mM ± 0.29 versus 3.93 mM ± 1.12; n = 12; p = 0.223). The response to glucose challenge at 10 weeks was comparable between wild type and transgenic males, with no difference in blood glucose concentration 30 minutes (12.24 mM ± 2.38 versus 11.57 mM ± 1.47; n = 12; p = 0.998) 60 minutes (11.38 mM ± 1.26 versus 12.69 mM ± 1.99; n = 12; p = 0.202) 90 minutes (9.74 mM ± 0.76 versus 11.03 mM ± 2.31; n = 12; p = 0.432) or 120 minutes (8.08 mM ± 0.80 versus 9.16 mM ± 1.54; n = 12; p = 0.343) following injection of glucose. At 6 months, there was also no difference in blood glucose concentration 30 minutes (12.46 mM ± 2.86 versus 10.94 mM ± 1.61; n = 12; p = 0.530) 60 minutes (13.66 mM ± 2.31 versus 12.31 mM ± 2.10; n = 12; p = 0.432) 90 minutes (11.56 mM ± 1.29 versus 10.61 mM ± 1.82; n = 12; p = 0.343) or 120 minutes (9.88 mM ± 0.90 versus 9.17 mM ± 1.50; n = 12; p = 0.202) after injection. Whereas at one year of age, transgenic females exhibited prolonged elevation of blood glucose, this was not observed in transgenic males, with no significant difference observed 30 minutes (9.84 mM ± 1.69 versus 10.10 mM ± 1.62; n = 12; p = 0.957), 60 minutes (9.28 mM ± 1.18 versus 9.70 mM ± 1.63; n = 12; p = 0.731), 90 minutes (8.50 mM ± 0.98 versus 8.57 mM ± 1.55; n = 12; p = 0.782) or 120 minutes (8.00 mM ± 0.54 versus 7.89 mM ± 1.49; n = 12; p = 0.554) after injection.

A measure of the efficiency of glucose clearance is the area under the curve (AUC) of the glucose tolerance test measured in arbitrary area units (AAU). No significant difference was observed for the area under the curve of glucose tolerance test for males at 10 weeks (1181.4 ± 128.6 versus 1242.0 ± 176.0; n = 12; p = 0.465), 6 months (1338.3 ± 185.8 versus 1203.0 ± 186.0; n = 12; p = 0.291) or 1 year (1006.5 ± 104.6 versus 1028.4 ± 158.6; n = 12; p = 0.935).

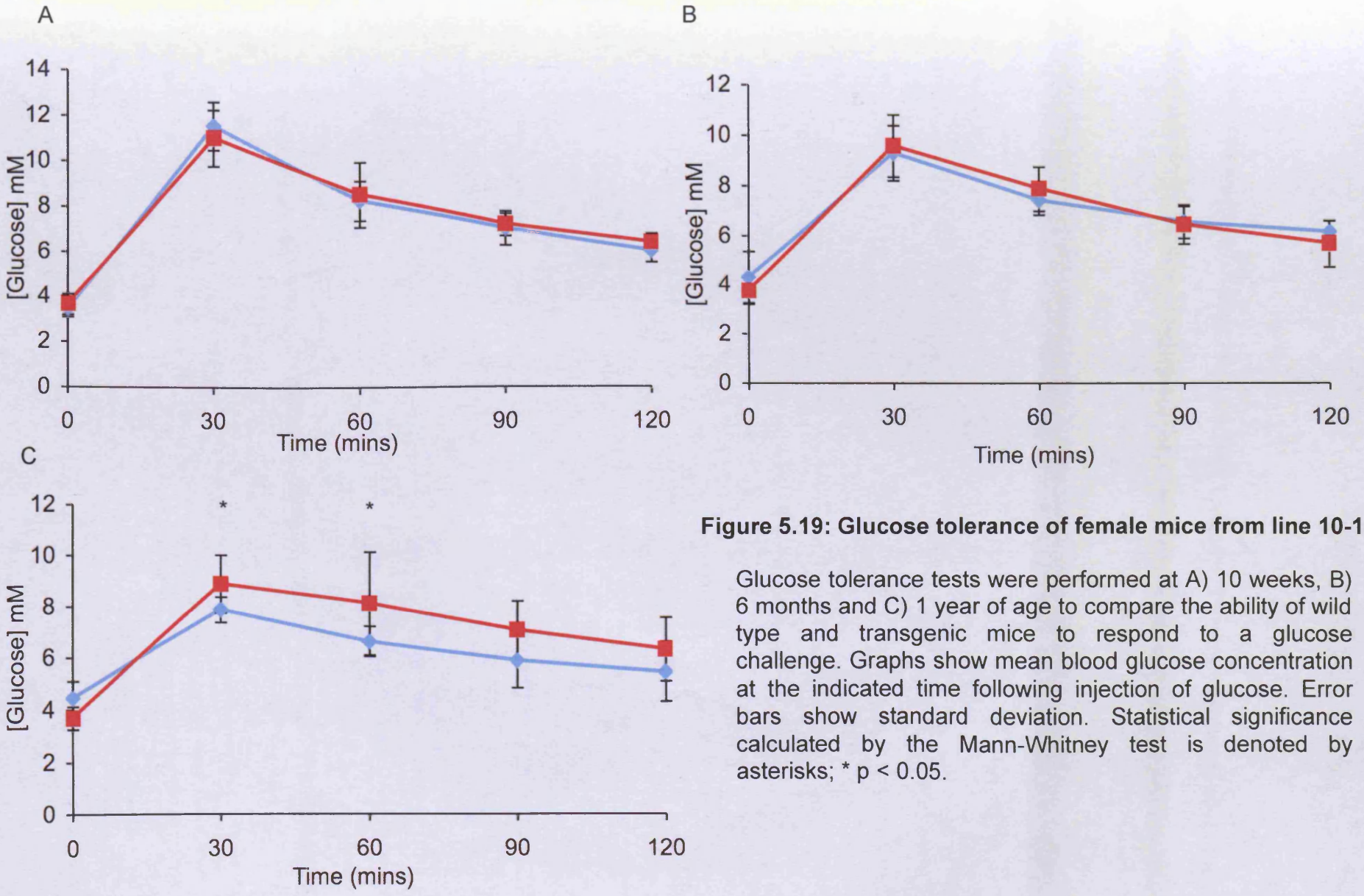


Figure 5.19: Glucose tolerance of female mice from line 10-15

Glucose tolerance tests were performed at A) 10 weeks, B) 6 months and C) 1 year of age to compare the ability of wild type and transgenic mice to respond to a glucose challenge. Graphs show mean blood glucose concentration at the indicated time following injection of glucose. Error bars show standard deviation. Statistical significance calculated by the Mann-Whitney test is denoted by asterisks; * $p < 0.05$.

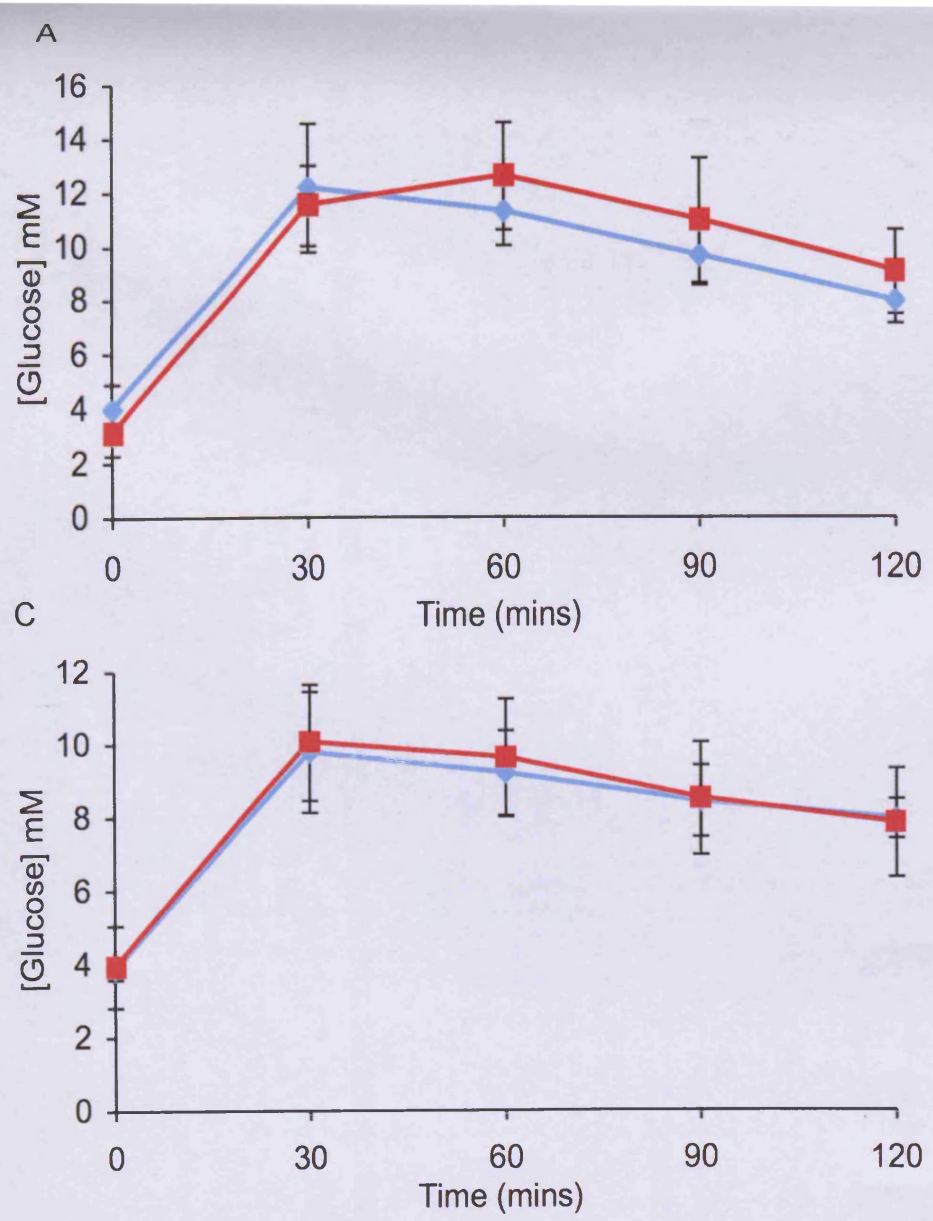


Figure 5.20: Glucose tolerance of male mice from line 10-15

Glucose tolerance tests were performed at A) 10 weeks, B) 6 months and C) 1 year of age to compare the ability of wild type and transgenic mice to respond to a glucose challenge. Graphs show mean blood glucose concentration at the indicated time following injection of glucose. Error bars show standard deviation.

Blood glucose levels are regulated primarily by the hormones insulin and glucagon. Insulin is released from the pancreas in response to elevated blood glucose, causing cells to take up glucose and store it as glycogen, thus reducing the circulating glucose concentration. Glucagon is secreted by the pancreas in response to low blood sugar levels, and causes the liver to convert stored glycogen into glucose, which is released into the blood. Type 2 diabetes is associated with reduced insulin sensitivity (insulin resistance) and impaired β-cell function. Insulin resistance means that normal levels of insulin do no elicit a sufficient insulin response, thus blood glucose levels remain elevated. Hyperinsulinemia often occurs in early stages of Type 2 diabetes as the pancreas continues to secrete insulin in an attempt to reduce blood glucose levels. Non-fasted serum insulin and glucagon concentrations were assessed by ELISA in females of 6 months and 1 year of age.

No difference was observed between wild type and transgenic non-fasted females for serum insulin concentration at 6 months (37.0 ρM ± 28.9 versus 28.9 ρM ± 13.7; n = 8; p = 0.886) or 1 year of age (33.0 ρM ± 16.4 versus 46.8 ρM ± 57.6; n = 11; p = 0.662) (Fig 5.21A). Similarly, no difference was observed for serum glucagon concentration at 6 months (6.2 ρM ± 2.2 versus 5.5 ρM ± 2.6; n = 8; p = 0.486) or 1 year of age (118.3 ρM ± 151.0 versus 61.0 ρM ± 40.0; n = 11; p = 0.792) (Fig 5.21B).

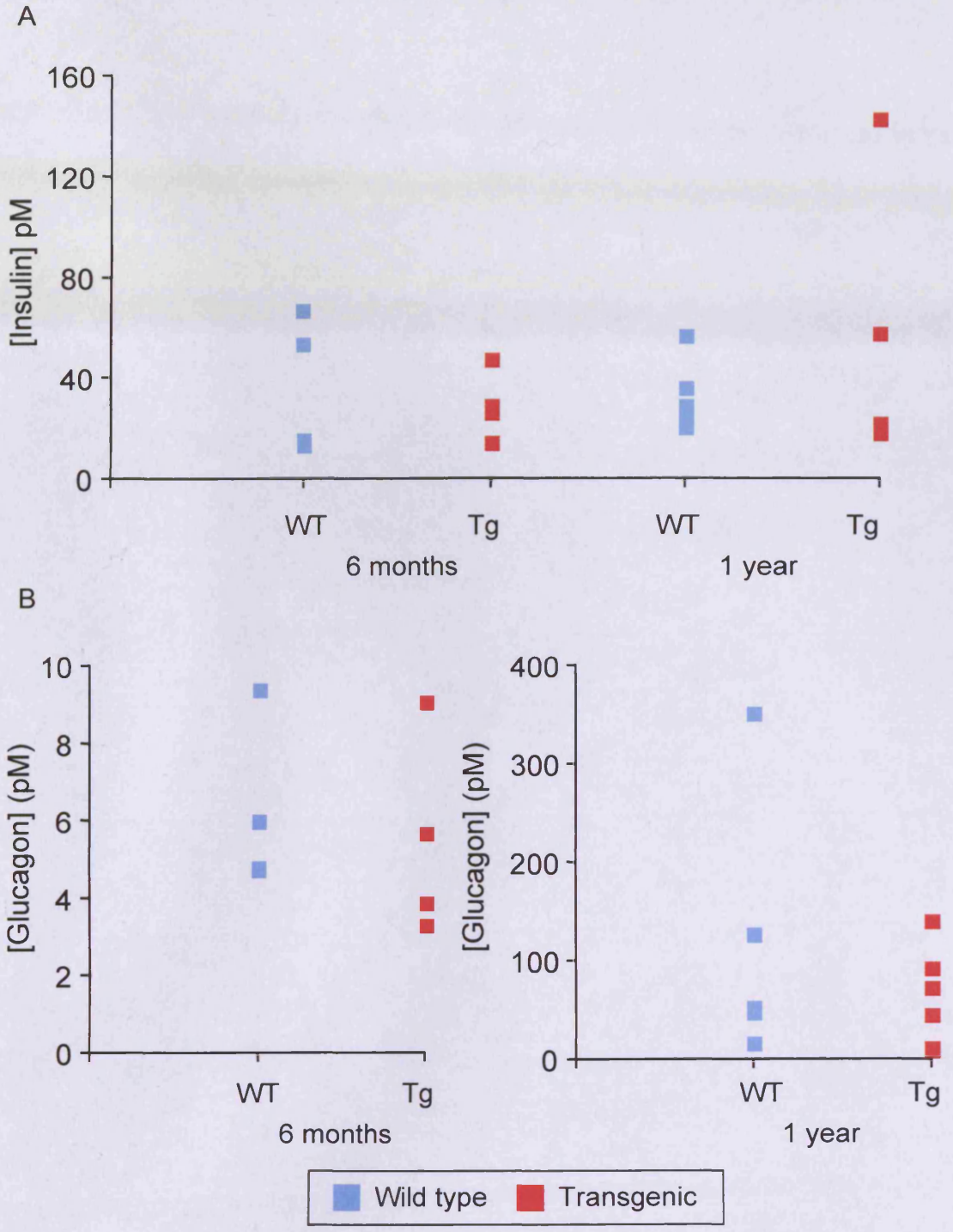


Figure 5.21: Insulin and glucagon serum concentrations

Serum insulin and glucagon concentration was quantified by ELISA at 6 months and 1 year of age from animals of line 10-15 on a pure 129/Sv background. Scatter graphs show individual serum hormone concentrations from female animals at the indicated ages.

5.2.7 Indication of perinatal lethality in transgenic animals

Low birth weight is associated with increased perinatal lethality (McIntire *et al.* 1999), whereas obesity and diabetes are known risk factors for reduced longevity in humans. There was no difference between the number of wild type and transgenic animals at any stage during gestation (Section 3.2.2). The number of wild type and transgenic animals observed at P0, P1 and weaning age were compared using the Chi-squared test to determine if the observed number differed from the expected number. At both P0 and P1 the calculated *p* value was < 3.841 (Table 5.1), thus indicating no difference in the number of wild type and transgenic animals. At weaning, the number of transgenic animals was significantly less than the number of wild type animals (136 versus 102; p = 4.857), although when the number of wild type and transgenic animals was compared by sex, no significant difference was observed for either females (73 versus 53; p = 3.175) or males (63 versus 49; p = 1.750). Furthermore, there was no difference in the number of male or female animals observed at P28 (112 versus 126; p = 0.824).

	WT	Tg	*P*
P0	79	75	0.104
P1	26	23	0.184
P28 males and females	136	102	4.857
P28 (females)	73	53	3.175
P28 (males)	63	49	1.750

Table 5.1: Analysis of number of wild type and transgenic neonates

Table shows *P* values for Chi-squared test for comparison of number of wild type and transgenic animals at P0, P1 and P28 from line 10-15 on a pure 129/Sv background.

	Females	Males	*P*
P28	112	126	0.824

Table 5.2: Analysis of number of male and females at weaning

Table shows *P* value for Chi-squared test for comparison of number of male and female animals at P28 from line 10-15 on a pure 129/Sv background.

5.2.8 Transgenic placentae exhibit elevated expression levels of nutrient transporters

Evidence presented thus far suggests that over-expression of *Phlda2* and *Slc22a18* in the mouse placenta causes placental insufficiency resulting in asymmetric IUGR of transgenic embryos with post-natal catch up growth complete within two weeks of birth. A similar growth profile is observed in mice that lack expression of placenta-specific *Igf2 (Igf2*P0 null*)*. Placental insufficiency results in onset of IUGR between E15.5 and E18.5, with transgenic animals born 30% lighter than wild type littermates. Transgenic animals display catch up growth that is complete by 3 months of age. The reduced size of *Igf2*P0 null placentae impairs the passive permeability, thus limiting the nutrient supply to mutant embryos (Constância *et al.* 2002). However, mutant placentae are able to compensate for their reduced size by upregulating expression of genes that encode nutrient transporter molecules. *Slc2a3*, which encodes a glucose transporter, and the 4-kb transcript from the imprinted gene *Slc38a4*, which encodes a member of the System A amino acid transporter family, are both upregulated in *Igf2*P0 null placentae at E15.5. Upregulation of these genes results in increased transport efficiency of mutant placentae, which is able to meet the nutrient demands of the embryo at this stage. However, System A transport was no longer upregulated at E18.5, and thus combined with the reduced passive permeability of the mutant placenta resulted in late gestation IUGR (Constância *et al.* 2005; Constância *et al.* 2002; Sibley *et al.* 2004).

Microarray analysis of gene expression for wild type and transgenic placentae of line 10-15 was performed by Ben Tycko. Gene expression was compared between three wild type and three transgenic placentae at E16.5. Eight genes encoding nutrient transporters were significantly upregulated in transgenic placentae and one transporter *Slc22a5* was significantly downregulated by nearly 5-fold (Table 5.4). Similar to the *Igf2*P0 null placentae, *Slc2a3* was upregulated, with one probe reporting a 1.7 fold upregulation and a second probe indicating a 2.9-fold upregulation. In contrast to the *Igf2*P0 model, no significant effect on expression level of *Slc38a4* was detected although it is unknown whether the probe was specific for the 4-kb transcript.

Gene	Fold Change
Slc2a3	1.7 *
Slc2a3	2.9 *
Slc3a2	1.5 *
Slc19a2	2.0 *
Slc22a5	- 4.8 *
Slc22a3	2.5 *
Scl25a3	1.7 *
Slc38a1	-1.1 ^{NS}
Slc38a2	2.0 *
Slc38a4	1.3 ^{NS}
Slc40a1	2.1 *
Slc41a1	1.7 *

Table 5.3: Relative expression levels of genes encoding placental transporters in transgenic placentae of line 10-15

Fold change of transporter expression in transgenic placentae at E16.5 determined by microarray. Statistical significance determined by Mann Whitney test is denoted by asterisks; * $p < 0.05$, ^{NS} = not significant.

Mice that completely lack expression of *Igf2* are inherently growth restricted, and do not exhibit altered placental gene expression, presumably as the mutant placenta is able to supply the lower nutrient demand of the mutant embryo (Constância *et al.* 2005). It is possible that the altered gene expression observed in transgenic placentae of line 10-15 is a direct result of the perturbed placental structure, and does not represent upregulation of gene expression to compensate for reduced placental function. In order to eliminate this possibility, gene expression was examined by qPCR in placentae of line 5D3. Results presented in Chapter 4 show that transgenic placentae of lines 5D3 and 5A4 exhibit a similar placental defect to line 10-15. Transgenic embryos of these lines additionally over-express the growth-inhibitory *Cdkn1c* in the embryo, resulting in intrinsic embryonic growth restriction from at least E13.5. Transgenic embryos of line 5D3 on a mixed 129/Sv x MF1 background were 14% lighter at E13.5, whereas no effect on embryonic growth was observed in line 10-15 at this stage (Andrews *et al.* 2007). Transgenic embryos of line 5A4 possess two copies of the unmodified transgene, and expresses *Cdkn1c* at greater levels than line 5D3 in the embryo. Embryonic weights were compared for lines 10-15, 5D3 and 5A4 at E13.5 and E18.5 (Fig 5.21).

As presented in Figure 5.1, no difference in embryonic weight was observed at E13.5 in line 10-15 on a pure 129/Sv background. Transgenic embryos of line 5D3 on a mixed 129/Sv x C57BL/6 genetic background were 4% lighter than wild type at E13.5, although without reaching statistical significance (151.5 mg ± 14.1 versus 145.3 mg ± 16.1; 5 litters; n = 47; $p = 0.171$), whereas previously they had been shown to be ~20% lighter (Andrews *et al.* 2007). Transgenic embryos of line 5A4 on a similar mixed genetic background were 20.5% lighter than wild type (146.5 mg ± 15.3 versus 116.4 mg ± 14.6; 7 litters; n = 50; $p = 1.43 \times 10^{-4}$). At E18.5, transgenic embryos of line 10-15 were 13.4% lighter than wild type, whereas those from line 5D3 were 8.6% lighter (1300.8 mg ± 67.4 versus 1188.6 mg ± 51.5; 7 litters; n = 59; $p = 2.09 \times 10^{-5}$), and those of line 5A4 were 17.1% lighter (1295.8 mg ± 54.1 versus 1074.6 mg ± 138.2; 7 litters; n = 54; $p = 1.18 \times 10^{-9}$).

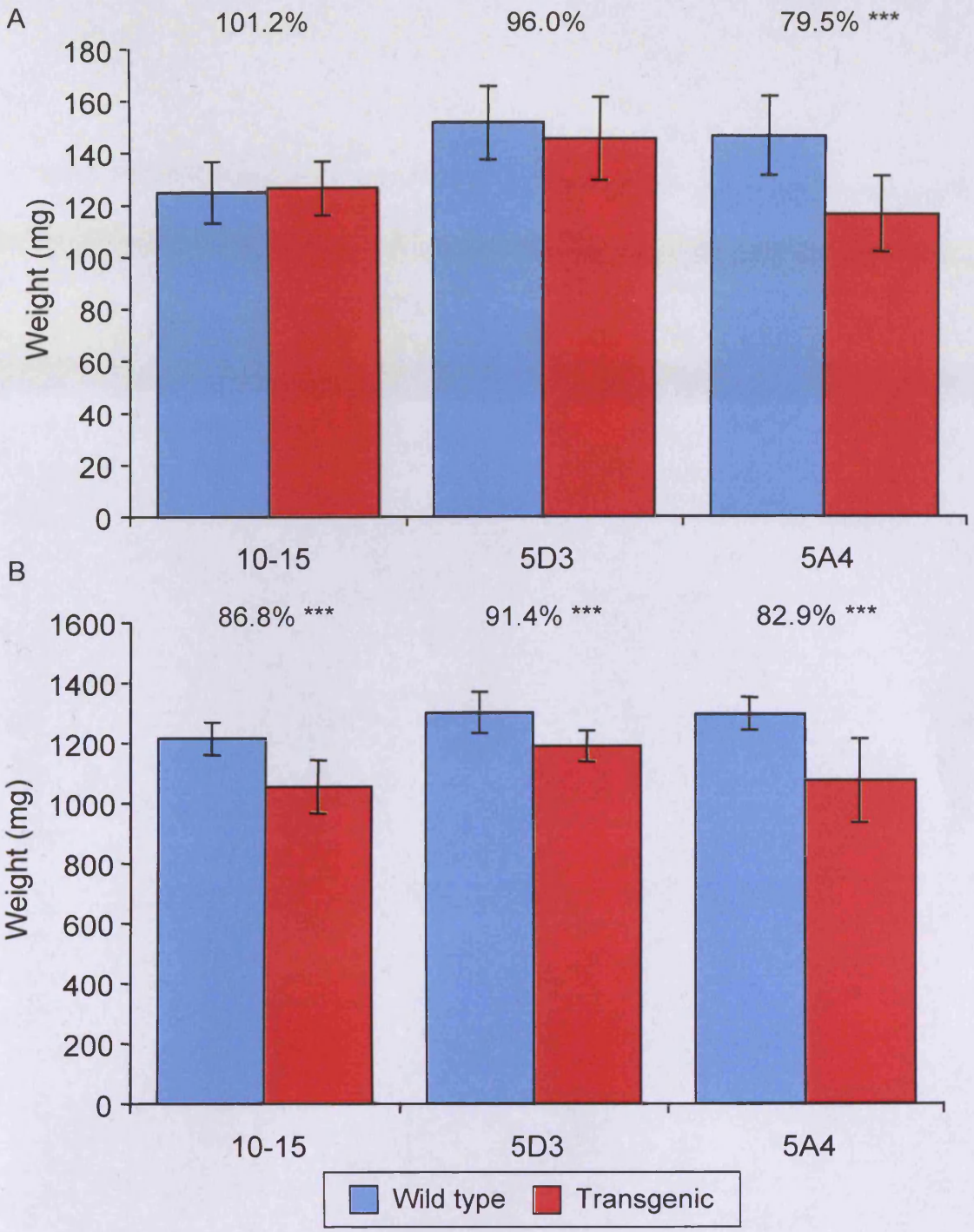


Figure 5.22: Embryonic growth restriction in transgenic lines 5D3 and 5A4

Graphs show embryonic growth of wild type and transgenic embryos in line 10-15 on a pure 129/Sv genetic background and lines 5D3 and 5A4 on the 129/Sv x C57BL/6 background at A) E13.5, and B) E18.5. Error bars represent standard deviation, with significance as determined by the *t*-test denoted by asterisks; * $p < 0.05$.

Expression of the glucose transporter *Slc2a3*, and the System A amino acid transporter *Slc38a4* was altered in *Igf2*P0 null placentae, whereas expression of another System A transporter *Slc38a2* was altered in *Igf2* null placenta. Expression of these three genes was thus also examined in placentae of lines 10-15 and 5D3. Additionally, expression of another molecule involved in amino acid transport, *Slc3a2*, was also compared between lines 10-15 and 5D3. *Slc3a2* (formerly *Cd98*) encodes a type II transmembrane protein that forms the heavy chain of a group of heterodimeric amino acid transporters (HATs) (Verrey *et al.* 1999; Verrey *et al.* 2000). Slc3a2 dimerises with members of the Slc7 family of light or catalytic chain HATs to mediate cationic and glycoprotein-associated amino acid transport (Verrey *et al.* 2004). Similar to the comparison of gene expression between the *Igf2*P0 null line and the *Igf2* null line, genes displaying similarly altered expression in both lines 10-15 and 5D3 lines were predicted to represent a direct effect of the disrupted placental structure. In contrast, those genes displaying upregulated expression only in transgenic placentae of line 10-15, and not line 5D3, were predicted to be a consequence of altered gene expression in order to compensate for reduced placental size.

Microarray analysis indicated that *Slc2a3* was upregulated by between 1.7- and 2.9-fold in transgenic placentae of line 10-15. Analysis of expression by qPCR confirmed that the fold change was around 1.7-fold wild type levels in line 10-15, with a similar degree of upregulation (1.6-fold) observed in line 5D3. *Slc3a2* expression was reported to be 1.5-fold wild type levels in microarray data, with qPCR analysis verifying this upregulation. In transgenic placentae of line 5D3, *Slc3a2* was expressed at 1.3-fold wild type levels. However, a direct comparison between fold change in line 10-15 and fold change in line 5D3 did not achieve statistical significance, indicating that there was no difference in the expression of *Slc3a2* in transgenic placentae of lines 10-15 and 5D3. A 2-fold increase in expression of *Slc38a2* was reported by microarray, with a 1.7 fold increase by qPCR. No significant difference in expression was found between transgenic and wild type placentae of line 5D3, with a significant difference between the fold change in line 10-15 and the fold change in line 5D3. Consistent with microarray data, no alteration to expression was observed for *Slc38a4* in line 10-15, whereas a 1.3-fold increase was observed for line 5D3, although this did not achieve statistical significance.

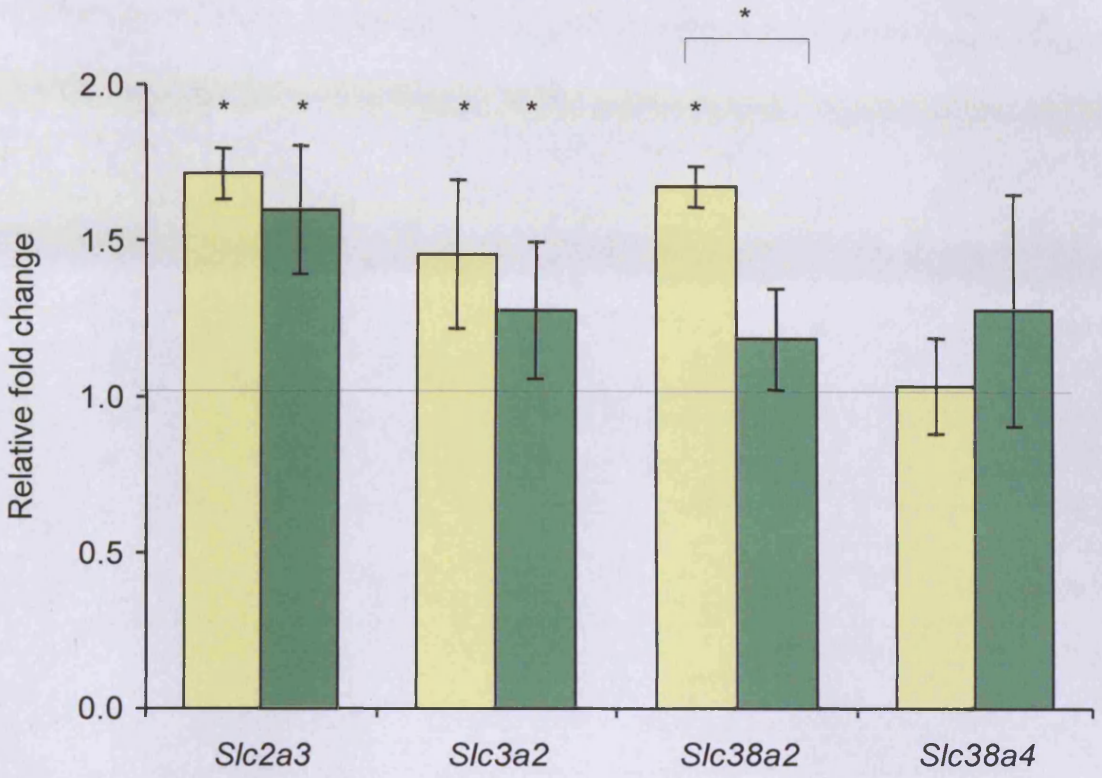


Figure 5.23: Relative expression levels of nutrient transporters in transgenic placentae

Graph shows the mean relative fold change of nutrient transporters in transgenic placenta of line 10-15 (yellow) and line 5D3 (green) compared to wild type at E16.5. Broken line indicates arbitrary expression level in wild type. Significance as determined by the Mann Whitney test is denoted by asterisks; * $p < 0.05$.

	10-15	5D3	*P*
Slc2a3	1.7 ± 0.08 ($p = 0.014$)	1.6 ± 0.21 ($p = 0.037$)	0.593
Slc3a2	1.5 ± 0.24 ($p = 0.014$)	1.3 ± 0.22 ($p = 0.034$)	0.154
Slc38a2	1.7 ± 0.07 ($p = 0.037$)	1.2 ± 0.16 ($p = 0.121$)	0.042
Slc38a4	1.0 ± 0.15 ($p = 1.00$)	1.3 ± 0.37 ($p = 0.487$)	0.439

Table 5.4: Relative expression levels of nutrient transporters in transgenic placentae

The table shows the mean values relating to the graph above, with the associated *p* value for the Mann Whitney test. The third column labelled "*P*" shows the *p* value for a comparison between fold change in transgenic placentae of lines 10-15 and 5D3.

5.3 Discussion

The primary aim of the work presented in this final experimental chapter was to ascertain the long-term consequences of the placental defect in transgenic mice that over-express *Phlda2* and *Slc22a18*. These investigations focused primarily on mice on a pure 129/Sv genetic background, as backcrossing of line 10-15 on to the C57BL/6 background had not progressed to generation six at the onset of the study. Embryonic growth was examined during gestation from the same litters that were used to investigate placental development. Neonatal animals were dissected on the day of birth (P0) to determine if organ weights of wild type and transgenic animals were proportional. A cohort of animals was weighed throughout the course of one year, with a subset of these animals used to ascertain fat pad weights and glucose tolerance at 10 weeks, 6 months and 1 year of age. Morphology of key organs, including adipose tissue and pancreas, was examined at 10 weeks and 6 months. Gene expression was also examined in transgenic placentae to assess whether the small placenta responded to embryonic nutrient demands by upregulating nutrient transporters as observed in *Igf2*P0 null conceptuses.

5.3.1 Placental defect and asymmetric IUGR is indicative of placental insufficiency restricting embryonic growth

Transgenic placentae from line 10-15 were significantly lighter than wild type at all gestational stages examined between E12.5 and E18.5. Previously published data reported a 10% weight deficit for transgenic embryos compared to wild type at E16.5, with no weight difference evident at E14.5 (Salas *et al.* 2004). Data presented in this investigation demonstrate a late onset and progressive embryonic growth restriction on a pure 129/Sv genetic background. No significant weight difference was observed at E14.5, whereas transgenic embryos were ~6% lighter at E16.5 and ~13% lighter at E18.5. Such late onset impaired embryonic growth is characteristic of placental insufficiency, whereby embryonic growth is adversely affected by an inability of the placenta to supply the required nutrients in late gestation (Brodsky and Christou 2004). This is consistent with the presence of a placental phenotype from at least E10.5 in line 10-15, with embryonic growth adversely affected in late gestation. This pattern of embryonic

growth indicates that the slow down in embryonic growth is not an intrinsic embryonic phenotype but is rather an indirect consequence of the placental defect. A similar placental defect and delayed embryonic growth phenotype exists in the *Igf2*P0 null mouse. Mutant placentae lack expression of placenta-specific *Igf2*, resulting in a significantly lighter placenta from at least E11.5. However, embryonic growth was only adversely affected after E15.5, with mutant embryos exhibiting a marginal weight deficit at this stage, a ~20% weight deficit at E18.5 and a ~30% weight deficit at birth (Constância *et al.* 2002). In this instance, only *Igf2* expression directed by the placental-specific P0 promoter was disrupted with normal embryonic *Igf2* expression maintained. This model clearly demonstrates the role of placental insufficiency in the resulting embryonic growth restriction.

The transgenic line utilised in this investigation possesses three additional copies of the maternally expressed *Phlda2* and *Slc22a18* imprinted genes. Although these genes are primarily expressed in the extraembryonic lineages, low-level expression of both genes also occurs in some embryonic tissues. For instance, *Phlda2* is expressed in the embryonic lungs, liver, kidney and limbs (Qian *et al.* 1997), whereas *Slc22a18* exhibits expression in embryonic liver, kidney and intestine (Dao *et al.* 1998). Due to the complication of elevated embryonic expression of the genes present on the transgene, it is not possible to eliminate an intrinsic effect for these genes on embryonic growth. However, considering the limited expression pattern, and the predicted functions of the genes, it seems unlikely that over-expression of these genes in a subset of embryonic tissues would cause such an overt embryonic growth deficit. Indeed, it seems more likely that the observed embryonic growth deficit observed in this transgenic line is a consequence of placental insufficiency. Prior to ~E14.5 the defective transgenic placenta is sufficient to support normal embryonic growth. However, from this stage, as embryonic nutrient demands surge in the final trimester of pregnancy, the transgenic placenta becomes unable to support the rapidly growing embryo. The placental insufficiency thus results in a moderate reduction in transgenic embryo weight at E16.5 progressing to a significant embryonic growth restriction effect with a ~13% weight deficit existing at E18.5.

Given that the spongiotrophoblast is not directly involved in nutrient transport and there is no overt labyrinth defect in the transgenic placenta, it seems unlikely that the reduced embryonic growth is directly attributable to

reduced placental nutrient transfer which occurs in the labyrinth layer. Considering the endocrine function of the spongiotrophoblast in releasing hormones to alter maternal physiology it is plausible that embryonic growth is restricted due to an inadequate modulation of maternal physiology by the transgenic placenta. As described in Chapter 3, there is an indication of reduced pregnancy specific glycoprotein transcription in transgenic placenta. Thus, the loss of spongiotrophoblast results in a reduction in pregnancy-related hormone secretion and a failure of the normal maternal adaptations that support late gestation embryonic growth. This may result in an insufficient blood supply to the placenta, thus limiting the availability of nutrient and gas exchange and indirectly restricting embryonic growth. This may also explain the elevated expression of *Slc38a2* that is observed in transgenic placenta at E16.5 (discussed in Section 5.3.4).

Additional support for the role of placental insufficiency in the observed embryonic growth restriction derives from recent reports of human IUGR data. Two recent publications have identified upregulated *PHLDA2* in the placenta of human IUGR and low birth weight infants (Apostolidou *et al.* 2007; McMinn *et al.* 2006). Furthermore, hypoxia induces down-regulation of *PHLDA2* in human term trophoblast cells, indicating that *PHLDA2* is a hypoxia responsive gene. This response to hypoxia of reducing *PHLDA2* expression would be predicted to allow increased placental growth thus minimising the effect of hypoxia (Kim *et al.* 2007). However, a failure of this hypoxia-responsive mechanism could cause placental insufficiency as an indirect consequence of inappropriate *PHLDA2* expression.

Transgenic pups were born approximately 11% lighter than wild type pups. However, considering the additional 10% weight deficit accrued by *Igf2*P0 null pups between E18.5 and birth, the absence of any additional weight deficit between these stages for this line seemed unusual. It was noted that some pregnant dams gave birth during the night as expected, with these litters collected at approximately 9 am. Other pregnant dams however gave birth during the day (E19.5), with these litters collected within a relatively shorter timeframe. Analysing the complete data set of P0 pups as two different data sets according to the time of birth revealed an interesting trend. The weight deficit of transgenic pups born during the night was relatively smaller than that of transgenic pups

born during the day (89% versus 87% respectively), although there was no statistically significant difference when transgenic weights were compared from the two data sets. Furthermore, the weight deficit of transgenic pups at P1 was reduced further (93%). By just one week of age, there was no statistically significant difference in weight between wild type and transgenic pups. This rapid post-natal catch up growth was further indicative of embryonic growth restriction due to placental insufficiency. Following the removal of the growth-limiting factor (the defective placenta) the transgenic embryo was able to re-establish and continue along its genetic growth trajectory. The post-natal catch up growth observed in this transgenic line was significantly more rapid than that seen in the *Igf2*P0 null line. *Igf2*P0 mutant embryos exhibit catch up growth that is complete within 3 months of birth (Constância *et al.* 2002), although the growth deficiency of both the placenta and embryo were more severe in the *Igf2*P0 null line.

Considering the evidence implicating placental insufficiency as the causative factor of the observed IUGR, organ weights of wild type and transgenic embryos were examined at P0 to determine if brain-sparing growth was present. Brain-sparing growth is characteristic of type II (asymmetric) IUGR, which would be predicted to occur in embryos whose growth has been restricted due to placental insufficiency in late gestation. In the cohort of animals for which organ weight was assessed, the degree of embryonic growth restriction affecting transgenic pups was ~11% at P0. A similar degree of growth restriction was observed on the liver, heart and kidneys of transgenic animals. In contrast, brain weight was only 5.5% less than wild type, whereas lungs were ~15% lighter and the pancreas ~20% lighter in transgenic pups. Expressing organ weights as a proportion of body weight revealed that the brain to body weight proportion of transgenic animals was nearly 7% larger than that of wild type animals. The ratio of organ to body weight for liver, heart and kidneys was not affected in transgenic pups, whereas the lungs and pancreas appeared to represent a smaller proportion of the body weight, although without achieving statistical significance. These data indicate that brain-sparing growth was evident in transgenic animals of line 10-15, thus suggesting the presence of asymmetric growth restriction.

Organ weights were also examined from embryos of line 5D3, which in addition to possessing a similar placental defect to line 10-15 also over-express *Cdkn1c* in a subset of embryonic tissues. As a consequence of this embryonic

over-expression of *Cdkn1c*, transgenic animals are intrinsically growth restricted, being born 10-30% lighter, depending upon genetic background, with no post-natal catch up growth evident (Andrews *et al.* 2007). Due to the inherently reduced growth potential of transgenic embryos of line 5D3, it is proposed that embryonic growth is not adversely affected by the presence of the placental defect. Consequently, transgenic embryos of line 5D3 would not be expected to exhibit asymmetric growth restriction. In contrast, transgenic embryos of this line would be predicted to be symmetrically growth restricted, due to the presence of a growth restrictive factor directly limiting embryonic growth. No significant difference in organ to body weight proportion was observed for any of the six organs examined from line 5D3, indicating the presence of symmetric growth restriction. However, the anticipated embryonic growth retardation of line 5D3 at P0 was less severe than predicted, with transgenic embryos only ~5% lighter than wild type at this stage.

This apparent loss of the embryonic growth phenotype in line 5D3 may be due to genetic background, with this experimental cohort generated on a mixed 129/Sv x C57BL/6 background, utilising transgenic males that were G7 C57BL/6. However, the embryonic growth phenotype was observed in line 5D3 at E18.5 on a mixed 129/Sv x C57BL/6 background, although utilising transgenic males that were from G6 C57BL/6. Additionally, the embryonic phenotype persists in line 5A4 on a similar mixed genetic background. Such evidence indicates that the loss of the embryonic growth phenotype is not attributable to the genetic background, but may instead be due to epigenetic inactivation of the transgene. Transgene inactivation is common in mice, with reports of inactivation of transgenes containing the globin genes (Robertson *et al.* 1995) or a *β-galactosidase* reporter (Montoliu *et al.* 2000), with this silencing apparently attributable to the CpG content of the inserted sequence (Chevalier-Mariette *et al.* 2003). Alternatively, the entire transgene may not be the subject of silencing, with *Phlda2* and *Slc22a18* remaining appropriately expressed from the transgene. Instead, the copy of *Cdkn1c* present on the transgene may be actively targeted and methylated in transgenic mice, thus silencing only this gene. Indeed, endogenous *Cdkn1c* accumulates post-fertilisation methylation of the promoter sequence in the paternal germline, which maintains monoallelic expression of this gene (Bhogal *et al.* 2004). Such a scenario would result in the persistence of the

placental phenotype indicating apparent integrity of expression from the transgene whereas no embryonic growth deficit would be seen. In an attempt to reverse the potential epigenetic modification of the transgene, the transgene will be passed through the female germline to re-establish transgenic males from which to generate experimental animals. In these animals, the presence of embryonic growth retardation will be examined in addition to an evaluation of gene expression levels in the embryo and placenta. If this approach fails, it may be necessary to introduce a degree of genetic heterogeneity by backcrossing for one generation onto the 129/Sv genetic background. However, this would prevent the subsequent generation of transgenic animals from line 5D3 on a pure C57BL/6 background.

5.3.2 Effect of genetic background on embryonic growth

Embryonic growth was examined on the 129/Sv x C57BL/6 genetic background for lines 10-15 and 10-10 at E14.5 and E18.5. At E14.5, no significant weight difference existed in either line 10-15 or 10-10 on this genetic background. At E18.5, transgenic embryos of lines 10-15 and 10-10 on the 129/Sv x C57BL/6 genetic background were significantly smaller than wild type. However, the degree of weight deficit was less than 50% of that observed on a pure 129/Sv genetic background. Transgenic embryos of line 10-15 were ~4% lighter and those of line 10-10 were ~6% lighter than wild type littermates at this E18.5, in contrast with the ~13% weight deficit of transgenic embryos from line 10-15 on a pure 129/Sv background.

Similar to wild type placenta on the 129/Sv x C57BL/6 genetic background, wild type embryos on this genetic background were significantly heavier than wild type embryos on a pure 129/Sv background. Although the extent of this weight difference was not as great as for placenta. Wild type embryos on the 129/Sv x C57BL/6 genetic background were ~10% heavier at E14.5 and ~8% heavier at E18.5. Whether the inherently heavier nature of embryos on this genetic background accounts for the reduced severity of the embryonic weight deficit is unclear. Alternatively, the less severe growth restriction may be due to the presence of an inherently larger spongiotrophoblast in placentae on the 129/Sv x C57BL/6 background. As discussed in Chapter 4, the midline spongiotrophoblast area of transgenic placenta from lines 10-15 and 10-10 on the 129/Sv x C57BL/6

genetic background was significantly larger than the midline spongiotrophoblast area of wild type placenta on a pure 129/Sv genetic background. Thus, a similar degree of spongiotrophoblast loss may not exhibit such an adverse effect on embryonic growth on the 129/Sv x C57BL/6 genetic background.

5.3.3 Asymmetric IUGR is associated with development of some aspects of metabolic syndrome

The immediate concern for human IUGR infants is the increased rate of neonatal lethality (Kok *et al.* 1998; McIntire *et al.* 1999). No embryonic lethality was observed for line 10-15, with the number of wild type and transgenic animals observed at P0 and P1 not deviating from the anticipated frequency. However, at weaning age there appeared to be significantly fewer transgenic animals than wild type animals from the combined numbers of males and females (136 wild type versus 102 transgenic; $\chi^2 = 4.857$). When these numbers were examined separately for males and females, although fewer transgenic animals appeared to be present in both instances, statistical significance was not achieved. Similarly, there was no significant difference in the number of male and female animals at P0 or P1. These data indicate a possible low frequency of neonatal lethality associated with this transgenic line.

In humans, IUGR followed by post-natal catch up growth is considered a risk factor for the development of metabolic syndrome in adulthood (Eriksson *et al.* 2000; Eriksson *et al.* 1999; Forsen *et al.* 2000; Forsen *et al.* 1999). Development of obesity and type 2 diabetes, two of the key factors of metabolic syndrome (reviewed in Alberti *et al.* 2005), were examined in a small cohort of mice from line 10-15. The glucose tolerance test was utilised to assess the ability of wild type and transgenic animals to respond to an elevated blood glucose concentration. Development of obesity was assessed by weighing a larger cohort of animals throughout the course of a year and by directly weighing adipose depots in a subset of animals at three time-points.

Transgenic mice did not develop overt obesity within one year of age, with no statistically significant weight difference between wild type and transgenic animals of either gender at this age. Although transgenic males were slightly heavier than wild type between 5 and 9 weeks of age, this weight difference did not persist and the biological significance remains unclear, possibly representing

an over-compensation of catch-up growth. No overt weight difference was observed between wild type and transgenic animals up to one year of age. However, transgenic females exhibited a progressive increase in adiposity, with a significant increase in the combined retroperitoneal and mesenteric adipose tissue weight at one year of age. No significant difference in adipocyte cell diameter was observed, suggesting instead a possible increase in cell number. However, adipose tissue from obese individuals may be composed of pockets of small adipocytes within regions of larger adipocytes (Ashwell *et al.* 1975). Although this was not immediately apparent from the sections examined, a more detailed examination of sections from a greater number of individuals would be needed in order to exclude this possible scenario.

In addition to the increased adiposity observed in female transgenic animals at one year of age, serum leptin levels were also significantly elevated in this cohort. Leptin is primarily produced from white adipose tissue (WAT) and acts to signal satiety and thus prevent excess food intake (hyperphagia). Mice lacking function leptin production are overtly obese, as are those with a disruption of the leptin receptor (Pelleymounter *et al.* 1995; Tartaglia *et al.* 1995). Leptin resistance may play a role in development of obesity, with mice unable to respond appropriately to the leptin produced by the adipose tissue (Frederich *et al.* 1995). To examine whether transgenic females of line 10-15 exhibit leptin resistance, it will be necessary to compare food intake with wild type mice over a period of time. Leptin resistance would present as hyperphagia, thus indicating an inability to respond appropriately to leptin signalling.

Glucose tolerance testing indicated a significantly impaired ability to respond to an elevated blood glucose level and may be indicative of Type 2 diabetes. Type 2 Diabetes may result from insulin resistance, impaired β-cell function or more usually a combination of both. Insulin tolerance testing may be performed to assess insulin response at similar stages to glucose tolerance testing. Similarly, β-cell function could be assessed by immunohistochemical staining of pancreas sections for insulin. Alternatively, pancreatic explant cultures could be utilised to assess insulin response *in vivo*.

It is clear that the experimental setup utilised in this investigation was not optimised to induce obesity, however the modest increase in adiposity observed

for some individuals is promising for future investigations. There are two general factors that must be considered when designing future experiments to further investigate development of obesity in these mice, these are the genetic background of the mice and the dietary provisions for the animals. Firstly, the adult phenotype was investigated using mice on a pure 129/Sv genetic background. This is generally considered to be genetically resistant to weight gain and glucose intolerance in comparison to more susceptible strains such as C57BL/6 (Leiter 1989). Backcrossing of line 10-15 to the C57BL/6 background is underway, with the intention of using mice of at least generation 12 on this background. Secondly, it is clear that obesity can only occur if energy intake exceeds energy expenditure. This can occur through numerous mechanisms, including increased food intake, reduced physical activity, altered metabolism or a combination of several factors. One of the most widely used animal models of obesity is the high fat diet (HFD) fed mouse. This model utilised wild type C57BL/6 mice fed a diet that contains five times as much fat and twice the calorie content of standard chow (Sorhede Winzell and Ahren 2004; Surwit *et al.* 1988).

A potential complication to further characterising the post-natal phenotype on a pure C57BL/6 genetic background is the apparently reduced severity of embryonic growth restriction on the 129/Sv x C57BL/6 background. Transgenic animals on a pure 129/Sv genetic background are born ~13% lighter and display only a modest increase in adiposity and decrease in glucose tolerance. Whether such phenotypes would manifest in animals that were born only ~4-6% lighter is unlikely. Therefore, prior to characterising the post-natal phenotype of line 10-15 on a pure C57BL/6 background, it will be necessary to determine the extent of growth restriction at birth on this genetic background. If the severity of embryonic growth restriction on the pure C57BL/6 background is not comparable to that on a pure 129/Sv background, it may be necessary to re-evaluate the use of the pure C57BL/6 background. Instead, the post-natal characterisation could be continued using mice on a pure 129/Sv genetic background, but fed a high fat, highly palatable diet as described above.

Investigation of the embryonic and post-natal phenotypes were complicated by the endogenous expression of *Phlda2* and *Slc22a18* in embryonic and adult tissues. For instance, both *Phlda2* and *Slc22a18* are expressed in adult kidney (Dao *et al.* 1998; Qian *et al.* 1997), with qPCR analysis of gene

expression showing over-expression of both genes in the kidney of transgenic animals (data not shown). The increased adiposity and reduced glucose tolerance reported here are indicative of aspects of metabolic syndrome occurring as a consequence of IUGR and catch-up growth. However, a direct causative role for the over-expression of *Phlda2* and/or *Slc22a18* in embryonic or adult tissues cannot be excluded at this stage. Such a role seems unlikely, given the restricted expression of these genes in embryonic and adult tissues. Furthermore, preliminary characterisation of line 5A4 found that a similar effect on adiposity and glucose tolerance was not observed in transgenic animals of line 5A4 (data not shown). Both *Phlda2* and *Slc22a18* remain over-expressed in the relevant tissues of transgenic embryos and adults of line 5A4, with these animals differing only in the presence of an intact *Cdkn1c* gene on the two copies of the transgene present. Thus, the absence of such a post-natal phenotype in line 5A4 suggests that over-expression of *Phlda2* and/or *Slc22a18* in embryonic or adult tissues is not directly causative of the observed post-natal phenotype.

5.3.4 Compensation for reduced placental size by upregulation of transporter expression

Placental transport efficiency was examined in *Igf2*P0 null conceptuses, which comprise an embryo with a normal growth potential but with severely reduced placental growth. Although the passive diffusion of mutant placentae was decreased, active transport of amino acids and glucose was increased at E15.5 due to upregulation of genes encoding a glucose transporter (*Slc2a3*) and a member of the System A amino acid transporter (*Slc38a4*) (Constância *et al.* 2002; Sibley *et al.* 2004). However, *Slc38a4* expression was no longer elevated at E18.5 and System A transport efficiency was reduced (Constância *et al.* 2005). The apparent compensatory upregulation of *Slc2a3* and *Slc38a4* at E15.5 was predicted to allow relatively normal growth of the mutant embryos up to this stage. The inability to maintain this increased transport efficiency to term combined with the reduced passive permeability of *Igf2*P0 null placenta resulted in embryonic growth restriction at E18.5 (Constância *et al.* 2005; Constância *et al.* 2002). In contrast, mice that lack embryonic *Igf2* expression in addition to the loss of placenta-specific *Igf2*, do not exhibit this compensatory increase in active transport capacity. This is predicted to be due to the reduced nutrient demand of

the *Igf2* null embryo, which is met, and possibly exceeded by the mutant placenta. Indeed at E18.5, expression of a second System A amino acid transporter gene *Slc38a2* was downregulated in *Igf2* null placenta (Constância *et al.* 2005; Constância *et al.* 2002).

The presence of a similar compensatory upregulation of nutrient transport associated genes was examined in transgenic placenta of line 10-15 at E16.5. Transgenic conceptuses of this line exhibit a comparable growth phenotype to *Igf2*P0 null conceptuses, with placental weight deficit evident from early gestation and late gestation embryonic growth restriction from ~E16.5 (Constância *et al.* 2002). Similarly, conceptuses of line 5D3 exhibit a comparable growth phenotype to *Igf2* null conceptuses, with an early gestation placental weight deficit and an intrinsic embryonic growth deficit (Andrews *et al.* 2007). Placental gene expression was compared between lines 10-15 and 5D3 at E16.5 to establish if any genes encoding nutrient transporters were upregulated potentially as a compensatory response to placental insufficiency. Those genes exhibiting increased expression in line 10-15 but remaining unaltered in line 5D3 were presumed to be altered in response to placental insufficiency. In contrast, those genes exhibiting similar expression patterns in transgenic placentae of lines 10-15 and 5D3 were presumed to exhibit perturbed expression due to the disrupted placental structure common to both lines. As established in Chapter 4, the placental phenotype of line 5D3 is comparable to that of line 10-15, thus allowing a comparison of gene expression between the two lines.

Initial microarray analysis of gene expression analysis was performed on placentae of line 10-15 at E16.5. A number of nutrient transporter genes were included on this array, the majority of which displayed significantly upregulated expression in transgenic placentae. Expression of *Slc2a3*, *Slc38a2* and *Slc38a4* was further assessed by qPCR from placentae of lines 10-15 and 5D3 at E16.5. In addition to these genes, which displayed perturbed expression in either the *Igf2*P0 null or *Igf2* null models, expression of *Slc3a2*, which encodes a further amino acid transporter, was also assessed by qPCR.

Similar to *Igf2*P0 null placentae, expression of the glucose transporter *Slc2a3* was upregulated in transgenic placentae of line 10-15. However, a similar degree of upregulation was also observed in transgenic placenta of line 5D3, with no significant difference between expression levels in transgenic placentae of

lines 10-15 and 5D3. This suggests that upregulation of *Slc2a3* is either an indirect consequence of the disrupted placental structure or that placental glucose transport is insufficient to meet the needs of transgenic 5D3 embryos.

The amino acid transporter *Slc38a4* was upregulated in *Igf2*P0 null placentae at E15.5 but not at E18.5 (Constância *et al.* 2005; Constância *et al.* 2002). However, no significant difference in expression of *Slc38a2* was detected by microarray analysis of placentae from line 10-15. Two transcripts exist for *Slc38a4*; one of 2-kb and the other of 4-kb in length, differing only in the extent of the 3'-UTR. In *Igf2*P0 null placenta, only expression of the 4-kb transcript was elevated, with expression of the 2-kb transcript unaffected. It is not known whether the microarray probe was specific to the 4-kb transcript or whether this probe detected both transcripts. Primers were designed to amplify a region of the 3'-UTR that is present only in the 4-kb transcript such that the expression of this mRNA could be specifically assessed by qPCR. However, no significant difference in expression was observed for transgenic placentae of either line 10-15 or 5D3.

Slc3a2 was upregulated 1.5-fold in line 10-15, with a 1.3-fold increase observed in line 5D3. However, similar to expression of *Slc2a3*, there was no significant difference between expression of *Slc3a2* in transgenic placentae of lines 10-15 and line 5D3. In contrast, expression of *Slc38a2*, a second member of the System A amino acid transporter complex, was upregulated in transgenic placentae of line 10-15 but unaltered in those of line 5D3. This pattern is somewhat in contrast to the altered expression in the two *Igf2* deficient models, with expression of *Slc38a2* unaltered in *Igf2*P0 null placentae but significantly downregulated in *Igf2* null placenta. Nonetheless, the upregulation of *Slc38a2* in line 10-15 but no effect in line 5D3 indicates that *Slc38a2* may be involved in the compensatory mechanism to alleviate the effect of placental insufficiency.

Four nutrient transporters were selected for further analysis of gene expression based upon the altered expression of three of these genes in *Igf2*P0 null placentae (Constância *et al.* 2005). Of these four genes, only *Slc38a2* was upregulated in transgenic placentae of line 10-15 with no effect on expression in transgenic placentae of line 5D3. This observation contrasts with the *Igf2*P0 null model in which expression of *Slc38a2* was unaltered, whereas expression of *Slc2a3* and *Slc38a4* was elevated at a similar stage of gestation. It cannot be

concluded as to whether the upregulation of *Slc38a2* occurs to compensate for reduced placental transport capacity. In order to further investigate this, it would be necessary to examine the active transport capacity of placentae from both lines 10-15 and 5D3 by using similar experiments to those described by Constância *et al.* (2005; 2002).

The apparently different effects on gene expression between the BAC transgenic mice described in this study and the *Igf2*P0 null mice reported previously may be due to the morphological differences between the placental defects of each line. *Igf2*P0 null placentae exhibit a proportionate reduction of both spongiotrophoblast and labyrinth layers (Constância *et al.* 2002), whereas over-expression of *Phlda2* and *Slc22a18* in the BAC transgenic mice is associated with a disproportionate loss of the spongiotrophoblast region. The different functions of these two layers may thus result in embryonic growth restriction through different mechanisms, and may therefore invoke alternative responses to alleviate placental deficiency. For instance, the labyrinth layer mediates nutrient transport, whereas the spongiotrophoblast is predicted to have a role in secreting hormones that alter maternal physiology. As discussed in Section 5.3.1, the disproportionate loss of the spongiotrophoblast layer in the BAC transgenic mice described in this investigation may be associated with an insufficient modulation of maternal physiology. This may indirectly restrict embryonic growth by reduced blood flow to the placenta, which may respond by upregulation of genes encoding nutrient transporters, such as *Slc38a2*. Interestingly, in contrast to the proportional loss of labyrinth and spongiotrophoblast layers of *Igf2*P0 null placentae, there is a disproportionate loss of the labyrinth layer in *Igf2* null placentae (Coan *et al.* 2008).

5.3.5 Summary of main findings

The primary aim of the work presented in this Chapter was to characterise embryonic growth and the post-natal consequences associated with the placental defect of line 10-15 described in Chapter 3. Transgenic embryos were significantly growth restricted from E16.5 with a birth weight ~13% less than that of wild type littermates. Embryonic growth restriction at E18.5 was less severe on a mixed 129/Sv x C57BL/6 background, possibly due to the presence of a larger spongiotrophoblast area in the associated placentae. Brain-sparing and rapid

post-natal catch up growth was evident in transgenic animals of line 10-15 on a pure 129/Sv background, with no significant weight difference between wild type and transgenic adult mice. Transgenic female mice displayed a progressive increase in adiposity over the course of a year with a significant reduction in glucose tolerance at one year of age. However, these symptoms were not observed in transgenic males.

Gene expression was examined in placentae of lines 10-15 and 5D3 at E16.5 to determine if any compensatory increase in nutrient transporter expression was present. Although expression of *Slc2a3* and *Slc3a2* was increased in transgenic placentae of line 10-15, a similar level of expression was observed in placentae of line 5D3, indicating that the altered expression was not to compensate for placental insufficiency. In contrast, expression of *Slc38a2* was upregulated in transgenic placentae of line 10-15, with no upregulation in line 5D3, indicating that at least one nutrient transporter molecule exhibits upregulated expression in line 10-15. In summary, the embryonic and post-natal phenotype associated with transgenic animals of line 10-15 were:

- Asymmetric growth restriction and rapid post-natal catch up growth
- Increased adiposity and decreased glucose tolerance at one year of age in female mice
- Upregulation of *Slc38a2* in transgenic placentae at E16.5

While these data suggest that this is a model of placental insufficiency, a role for embryonic expression of *Phlda2* and/or *Slc22a18* cannot be excluded without generating additional transgenic models.

5.3.6 Future directions

The post-natal characterisation performed as part of this investigation utilised only a small cohort of animals on the 129/Sv genetic background. Although an indication of increased adiposity and reduced glucose tolerance was observed in female transgenic mice at one year of age, similar results were not observed for male animals at this age. Similarly, adiposity was not significantly altered in the four transgenic animals examined by MRI. Nonetheless, the manifestation of such symptoms in some transgenic animals warrants further investigation. As discussed in Section 5.3.3, the experimental set-up was not optimally designed to invoke the most severe phenotype. Future characterisation of the post-natal

phenotype of line 10-15 will be investigated on a pure C57BL/6 genetic background, which displays increased susceptibility to development of diabetes and obesity. Furthermore, to further induce weight gain, both wild type and transgenic animals will be fed a high fat, highly palatable diet, such as that described by Ozanne *et al.* (2004). Development of metabolic syndrome in transgenic animals of line 10-15 could also be further examined in line 10-10. Additionally, glucose tolerance and adiposity could be investigated in lines 5A4 and 5D3, although the over-expression of *Cdkn1c* in a subset of tissues would need to be considered in such characterisation. Indeed, preliminary data indicates that such aspects of metabolic syndrome are absent from transgenic animals of line 5A4. This observation may potentially exclude a role for embryonic or adult expression of *Phlda2* and/or *Slc22a18* in the post-natal phenotype reported for line 10-15. Although it is possible that the excess embryonic *Cdkn1c* in line 5A4 may obscure the effects of excess *Phlda2* and *Slc22a18*.

A further opportunity for future investigation is the altered gene expression of transgenic placentae. Microarray analysis revealed a large number of genes encoding nutrient transporter molecules that displayed altered expression at E16.5 in line 10-15. Expression of each could be further assessed by qPCR in line 10-15 and additionally in line 5D3 based upon the same principles described in Section 5.2.8. A comparison with expression in placentae of line 5A4 could also be performed, embryos of which are more severely growth retarded than those of line 5D3 but with a similar placental phenotype to line 10-15. Embryos of line 5A4 would thus be predicted to exert further reduced nutrient demand on the placenta than those of line 5D3. Furthermore, although identifying nutrient transporter genes that are upregulated in 10-15 but not in 5D3 or 5A4 may indicate increased transport efficiency, this cannot be concluded from such analysis. Instead, to confirm that the altered gene expression indeed results in an increase in transport efficiency, solute transport would need to be directly measured as described previously (Constância *et al.* 2005; Constância *et al.* 2002).

Chapter 6:

Final discussion

6.1.1 Summary of phenotypic consequences of over-expression of *Phlda2* and *Slc22a18* in the mouse placenta

The effect of excess expression of imprinted genes has not been extensively studied. However, such models are useful in attempting to elucidate the potential advantages of imprinting and monoallelic expression of particular genes. This work utilised a novel transgenic mouse model to characterise the consequences of excess expression of *Phlda2* and *Slc22a18* on mouse development. Previously, transgenic placentae were investigated on a mixed genetic background. Expression levels of both *Phlda2* and *Slc22a18* were more than 10-fold greater than endogenous levels, with transgenic placenta significantly lighter at E14.5 and E16.5. A comparison of the placental phenotype between *Kvdmr1* null and *Kvdmr1* / *Phlda2* double null conceptuses revealed that the placental weight deficit was due to a disproportionate loss of the spongiotrophoblast layer (Salas *et al.* 2004). This placental weight deficit of transgenic placenta was reciprocal to that of *Phlda2* null placenta, which exhibited placentomegaly due to an expansion of the spongiotrophoblast (Frank *et al.* 2002).

This work sought to build upon the published work by performing a more detailed characterisation of placentae from line 10-15 on a pure 129/Sv background. In contrast to the 10-fold wild type expression of *Phlda2* and *Slc22a18* reported for this line on a 129/Sv x C57BL/6 genetic background, expression levels were found to correlate with transgene copy number on a pure 129/Sv background. Despite this relative decrease in *Phlda2* and *Slc22a18* expression, placental stunting persisted to a similar degree. Transgenic placentae were 10-25% lighter than wild type from E12.5 until term, with the least severe weight deficit observed at E18.5. Transgenic placentae were characterised by a disproportionate loss of the spongiotrophoblast layer throughout gestation, with a complete mislocalisation of *Tpbpa*-positive cells at E18.5. Glycogen cell staining was reduced throughout gestation, with an apparent failure of glycogen cell migration at E16.5.

The placental phenotype of line 10-15 was also examined on the 129/Sv x C57BL/6 genetic background. Expression of *Phlda2* and *Slc22a18* remained consistent with transgene copy number on this genetic background. Additionally, the placental weight deficit observed at E14.5 on a pure 129/Sv genetic

background was comparable to that observed on the 129/Sv x C57BL/6 genetic background. However, this weight deficit persisted to a similar degree at E18.5 on the 129/Sv x C57BL/6 background, whereas the weight deficit was reduced to less than 10% on a pure 129/Sv background. Transgenic placentae were characterised by a disproportionate loss of the spongiotrophoblast region, although the mislocalisation of *Tpbpa*-positive cells at E18.5 appeared to be less severe than on the pure 129/Sv genetic background. The difference in expression levels observed reported from the previous study on a mixed genetic background and the current data using the 129/Sv x C57BL/6 genetic background cannot be easily explained. However, it is possible that the variable genetic background of line 10-15 over this time period had an effect. Line 10-15 was initially generated by pronuclear injection into F_1 C57BL/6 x CBA embryos and subsequently bred onto the 129/Sv genetic background. Initial characterisation of this line was performed following approximately 6 generations of breeding into the 129/Sv background with a further 6 generations of breeding performed prior to beginning this investigation.

The placental phenotype of a second, independent transgenic line was also characterised. Line 10-10 was generated as part of this investigation with qPCR analysis of gene expression suggesting the presence of 1-2 copies of the transgene. Line 10-10 was characterised only on the 129/Sv x C57BL/6 genetic background. The placental phenotype of line 10-10 was comparable to that of line 10-15 on a similar genetic background, with a comparable degree of placental stunting observed at both E14.5 and E18.5. Similarly, a disproportionate loss of the spongiotrophoblast layer was observed, with the mislocalisation of *Tpbpa*-positive cells within the labyrinth comparable to line 10-15 on the 129/Sv x C57BL/6 background.

The placental phenotype was examined in two additional transgenic lines that possess either one or two copies of an unmodified version of the transgene. Transgenic mice of these two lines possess active transgenic copies of *Cdkn1c*. Enhancer elements required for placental and embryonic expression in some tissues were absent from this transgene, thus isolating the consequences of excess *Phlda2* and *Slc22a18* in the placenta but not the embryo (John *et al.* 2001). These transgenic lines were characterised on the 129/Sv x C57BL/6 genetic background, and showed a comparable placental defect to lines 10-15

and 10-10 on a similar genetic background. However, the placental weight deficit of the two-copy line was more severe than that of the single copy line, which was itself slightly more severe than the weight deficit of lines 10-15 and 10-10.

The single copy unmodified BAC transgenic line was crossed with the *Phlda2* null line in an attempt to assess the relative contribution of over-expressing *Slc22a18* to the observed placental phenotype. Despite *Phlda2* expression remaining slightly elevated relative to wild type placentae, placental weight of double *Phlda2* null / 5D3 transgenic placentae was restored to within the normal range at E14.5. Similarly, spongiotrophoblast area was significantly increased in double transgenic placentae compared to 5D3 transgenic placentae, although remaining somewhat reduced in comparison to wild type. This may be because *Phlda2* expression was not fully restored to wild type levels or due to the difficulty of isolating placentae of a similar developmental stage when *Phlda2* expression is rapidly downregulated. Alternatively, as discussed previously, this may reflect an effect of the intrinsically small 5D3 transgenic embryo on restricting placental development.

Embryonic growth was significantly reduced from E16.5, with transgenic animals born approximately 13% lighter than wild type littermates. These animals displayed asymmetric growth restriction, characterised by relative sparing of brain growth and subsequently exhibiting rapid post-natal catch up growth that was complete within two weeks of birth. In contrast, transgenic animals of line 5D3 were intrinsically small and displayed no brain sparing or post-natal catch up growth. This late gestation embryonic growth restriction associated with a severe placental weight deficit from early gestation was indicative of IUGR as a consequence of placental insufficiency.

Based on epidemiological investigations in humans, low birth weight that is associated with rapid post-natal catch up growth is considered a risk factor for the development of aspects of metabolic syndrome in adult life (Eriksson *et al.* 1999; Forsen *et al.* 2000). Development of obesity and diabetes was monitored in a small cohort of animals from line 10-15 over the course of one year. Although known contributory factors such as a high fat diet were excluded from the investigation, female transgenic mice displayed increased adiposity and reduced glucose tolerance at one year of age. Transgenic animals from line 10-15 thus appear to undergo asymmetric IUGR as a consequence of an early gestation

placental defect. These animals display rapid post-natal catch up growth and display evidence of metabolic syndrome in adulthood. This investigation describes preliminary evidence that may establish line 10-15 as a potential genetic model of IUGR in which to further study fetal programming of metabolic syndrome.

6.1.2 Identifying the role of *Phlda2* in placental development

There are two possible approaches that may be taken in the characterisation of imprinted genes. The first is to generate loss of expression models by specifically disrupting the actively expressed allele. Such models provide insight as to the function of an imprinted gene product. For instance, loss of *Phlda2* results in placentomegaly as a consequence of a disproportionate expansion of the spongiotrophoblast layer, thus identifying a definitive role for *Phlda2* in regulating placental development (Frank *et al.* 2002). An alternative approach is to characterise over-expression models, in which expression of an imprinted gene is restored to or above the biallelic level. Such models provide additional insight as to the rationale for imprinting a particular gene. BAC transgenic mice possessing additional copies of the imprinted genes *Phlda2* and *Slc22a18* were characterised in this investigation. These mice displayed a severe placental defect that was evident from as early as E10.5. A major role for *Slc22a18* was excluded following the rescue of the placental phenotype after restoration of *Phlda2* gene dosage to normal. Over-expression of *Phlda2* was associated with a disproportionate loss and late gestation mislocalisation of the spongiotrophoblast layer. Furthermore, glycogen cell staining was significantly reduced throughout gestation, with an apparent failure of glycogen cell migration at E16.5. Microarray analysis of gene expression at E16.5 revealed a significant reduction in the glycogen cell marker *Gjb3*. Additionally, a reduction in the expression level of genes encoding enzymes involved in the synthesis and turnover of glycogen including *Gpil* and *Pygl* was also observed (data not shown). In contrast, expression of genes including *Prl2b1*, *Prl2c2*, *Prl7a2*, *Prl8a6* and *Prl8a8*, which specifically mark the spongiotrophoblast sub-population of cells (Simmons *et al.* 2008), was unaffected (data not shown). It therefore seems that *Phlda2* directly regulates the glycogen cell population, with over-expression of *Phlda2* apparently associated with a marked decrease in this specific sub-population of cells. In

order to confirm this hypothesis it will be necessary to quantify the abundance of both spongiotrophoblast sub-populations in wild type and transgenic placentae of line 10-15.

A comparable placental phenotype was observed in transgenic lines possessing between one and three copies of the transgene, thus indicating that the severity of the placental phenotype was unaffected by a gene dosage above two-fold. Biallelic expression of *Phlda2* would thus be associated with a lighter placenta with a smaller spongiotrophoblast and a reduced abundance of glycogen cells. Imprinting and monoallelic expression of *Phlda2* would be predicted to enable the formation of a larger placenta with an increased abundance of glycogen cells. The exact function of glycogen cells is unclear, although it has been proposed that they may serve as an energy source for embryonic growth during late gestation (Coan *et al.* 2006). Thus, a placenta that contains an increased abundance of glycogen cells may be better able to support increased embryonic growth. However, as discussed in the subsequent section, an increased placental capacity does not necessarily impart any growth advantage to the embryo, thus explaining the imprinting of the IC2 sub-domain rather than *Phlda2* in isolation.

6.1.3 Genomic imprinting and nutrient supply and demand

The majority of imprinted genes described to date are critically involved in the regulation of placental development (reviewed in Coan *et al.* 2005). Indeed, evolution of the eutherian placenta and the phenomenon of genomic imprinting appear to be phylogentically linked (Kaneko-Ishino *et al.* 2003). For instance, imprinting has not been described in monotremes, whereas *Igf2* is paternally expressed and *Igf2r* is maternally expressed in both eutheria and marsupials (Killian *et al.* 2000; Nolan *et al.* 2001; Suzuki *et al.* 2005). In contrast, the *Kvdmr1* locus appears to have become imprinted following the divergence of eutheria from marsupials, with *Cdkn1c* biallelically expressed in marsupials (Ager *et al.* 2008; Suzuki *et al.* 2005). The key difference between marsupials and eutherian mammals is the ability to support longer gestation periods and give birth to mature progeny (Killian *et al.* 2001; Renfree *et al.* 2008). The ability to support increased gestation and produce developmentally advanced progeny is linked with the evolution of an invasive placenta in eutherian mammals compared with

the more primitive non-invasive marsupial placenta (John and Surani 2000). Thus parental imprinting of specific genes appears central to the development of the eutherian placenta and consequently the ability to give birth to developmentally mature progeny.

The primary role of most imprinted genes appears to be in the control of embryogenesis, whether through regulation of the development of the embryo, placenta or both (Coan *et al.* 2005; Isles and Holland 2005; Tycko and Morison 2002). The placental conflict hypothesis suggests that imprinting arose due to the conflicting interests of the parental genomes during embryogenesis, with paternal interests lying solely in producing large, healthy and viable offspring whereas the mother must additionally maintain sufficient resources for her own survival and that of future progeny (Moore and Haig 1991). Thus, the maternal genome would favour the repression of growth promoting genes and the expression of growth restrictive genes whereas the paternal genome would favour expression of growth promoting genes and repression of growth restrictive genes. Indeed, the majority of imprinted genes investigated to date correlate with this prediction, with paternally expressed imprinted genes tending to be growth promoting and maternally expressed imprinted genes growth restrictive (Isles and Holland 2005; Tycko and Morison 2002).

Imprinted genes are proposed to play an important role in regulating embryonic demand for nutrients in addition to controlling the potential placental supply of these nutrients. Thus, imprinting of specific genes achieves a balance between fetal demand and placental supply of nutrients in order to achieve optimal embryonic growth. If there is an imbalance between the supply and demand of nutrients, then optimal embryonic growth can not achieved (Angiolini *et al.* 2006) For instance, loss of expression of *Phlda2* results in placentomegaly but no growth advantage is conferred to the embryo (Frank *et al.* 2002). This is likely due to the absence of an increased embryonic demand such that even with the potential increase in placental function, *Phlda2* null embryos do not benefit, as their genetic growth potential remains unaltered. The inverse relationship exists in the *Igf2*P0 null mouse, in which placental growth is severely reduced and indirectly restricts embryonic growth (Constância *et al.* 2002). In this case, the embryonic growth potential is unaltered, but the inability of the placenta to deliver the required nutrients results in growth restriction during late gestation.

The transient increase in placental transport efficiency and post-natal catch up growth of these mice supports the proposal that the mutant placenta is the growth-limiting factor. In contrast, additional loss of *Igf2* expression in the embryo is associated with a reduction in the genetic growth potential of the embryo that places fewer demands on the placenta, which thus does not increase transport efficiency and adult mice remain significantly smaller than wild type (Constância *et al.* 2005; Constância *et al.* 2002). Interestingly, maternal inheritance of a *H19* deletion results in embryonic and placental overgrowth. This is likely a consequence of the elevated placental and embryonic *Igf2* expression, which increases the genetic growth potential of the embryo in addition to the supply capacity of the placenta (Angiolini *et al.* 2006; Leighton *et al.* 1995).

As described above, embryonic growth is only enhanced if the placental supply capacity is associated with an increased embryonic growth potential. In the absence of increased embryonic demand, a larger placenta has no obvious benefit to the embryo. Likewise, an embryonic growth potential that exceeds the potential transport capacity of the placenta confers no advantage. However, in such instances the placenta may be able to adapt and transiently increase its transport efficiency, although this appears to be insufficient to support the growth potential of the embryo. Thus, imprinting of specific loci would only confer an embryonic growth advantage if the result were to increase placental transport capacity in addition to the genetic growth potential of the embryo.

The IC2 sub-domain of mouse distal 7 contains a number of maternally expressed imprinted genes in addition to the paternally expressed *Kcnq1ot1*, which is associated with repression of these genes on the paternal chromosome (Mancini-Dinardo *et al.* 2006; Thakur *et al.* 2004). The IC2 sub-domain is a major regulator of embryonic and placental development. Paternal deletion of *Kvdmr1* results in biallelic expression of the maternally expressed imprinted genes within the domain and as predicted by the parental conflict hypothesis is associated with reduced embryonic and placental growth (Fitzpatrick *et al.* 2002; Salas *et al.* 2004). Similarly, mice that lack expression of *Cdkn1c* exhibit placentomegaly with a proportionate expansion of labyrinth and spongiotrophoblast layers (Takahashi *et al.* 2000a). Embryonic growth was enhanced at E13.5 (Andrews *et al.* 2007), although was severely disrupted towards term, with few animals surviving the neonatal period, indicating the crucial role of *Cdkn1c* in proper embryonic

development (Takahashi *et al.* 2000b; Yan *et al.* 1997; Zhang *et al.* 1997). Mice lacking expression of *Phlda2* also displayed placentomegaly, with a disproportionate expansion of the spongiotrophoblast (Frank *et al.* 2002). In contrast, loss of *Ascl2* expression results in a smaller placenta due to a loss of the spongiotrophoblast layer, with precursor cells instead differentiating into giant cells (Guillemot *et al.* 1994). Examination of the phenotype of mice deficient for a specific gene product provides insight as to the role of a specific gene. However, it is also important to consider the phenotype associated with over-expression of imprinted genes. Such models can provide insight as to the scenario prior to imprinting of a specific locus and thus identify potential advantages for favouring monoallelic expression of these genes.

We recently reported the intrinsic embryonic growth restriction associated with over-expression of *Cdkn1c* in only a subset of embryonic tissues (Andrews *et al.* 2007). Similarly, as reported previously (Salas *et al.* 2004) and further characterised in this investigation, over-expression of *Phlda2* is associated with a placental defect that is associated with embryonic growth restriction. Thus, biallelic expression of *Cdkn1c* and *Phlda2* would be predicted to be associated with smaller, less efficient placenta, and in the case of *Cdkn1c*, a reduced embryonic growth potential.

The structural organisation of the mouse distal 7/human 11p15.5 domain predates imprinting of this locus (Paulsen *et al.* 2005), with imprinting evidently established following the divergence of eutheria and marsupial (Suzuki *et al.* 2005). Biallelic expression of genes in the mouse IC2 sub-domain results in a 20-25% reduction in embryonic and placental growth (Fitzpatrick *et al.* 2002; Salas *et al.* 2004), clearly demonstrating a role for imprinting this domain in achieving increased placental and embryonic growth.

Figure 6.1 depicts the rationale for imprinting the entire IC2 sub-domain rather than *Phlda2* in isolation. Prior to imprinting at the domain, biallelic expression of *Phlda2* and *Cdkn1c* meant that embryonic growth was genetically limited with sufficient nutrients supplied by a similarly small placenta. In this scenario, embryonic demand and placental supply for nutrients was balanced, resulting in the development of a genetically small embryo that achieved its growth potential. The theoretical imprinting and the subsequent monoallelic expression of *Phlda2* in isolation would result in the development of a larger

placenta, but have no growth advantage on the embryo due to the lack of increased nutrient demand. Similarly, imprinting and monoallelic expression of *Cdkn1c* in isolation would also theoretically result in a larger placenta. However, in this scenario the genetic growth potential of the embryo would also be enhanced. It is plausible that the increased placental capacity achieved by imprinting of *Cdkn1c* would be insufficient to meet the increased embryonic growth potential associated with monoallelic expression of *Cdkn1c*. Indeed, *Cdkn1c* null embryos are not significantly heavier than wild type at birth (Takahashi *et al.* 2000b; Yan *et al.* 1997; Zhang *et al.* 1997). A similar situation is observed in the maternal inheritance of a *H19* deletion. In these conceptuses, the resulting ~30% increase in placental weight is only associated with a ~12% increase in embryonic growth (Angiolini *et al.* 2006; Leighton *et al.* 1995). Thus, a larger placenta would be required in order for embryos with monoallelic expression of *Cdkn1c* to achieve their genetic growth potential. This would be achieved by imprinting of the IC2 sub-domain, with monoallelic expression of *Phlda2* and *Cdkn1c* increasing the genetic growth potential of the embryo and additionally providing a placenta sufficient to support such growth.

It will be interesting to identify the enhancers required for placenta-specific *Cdkn1c* expression and generate BAC transgenic mice that over-express *Cdkn1c* in the placenta in addition to the full complement of embryonic tissues. Additionally, it may be possible to specifically disrupt the embryonic enhancer elements thus generating transgenic mice that over-express *Cdkn1c* in the placenta only. Such mice would be predicted to be comparable to the *Igf2* and *Igf2*P0 null models respectively. Embryonic and placental over-expression of *Cdkn1c* would be predicted to result in a balanced reduction of embryonic and placental growth, whereas over-expression of *Cdkn1c* in the placenta alone would create an imbalance, with only placental growth directly reduced.

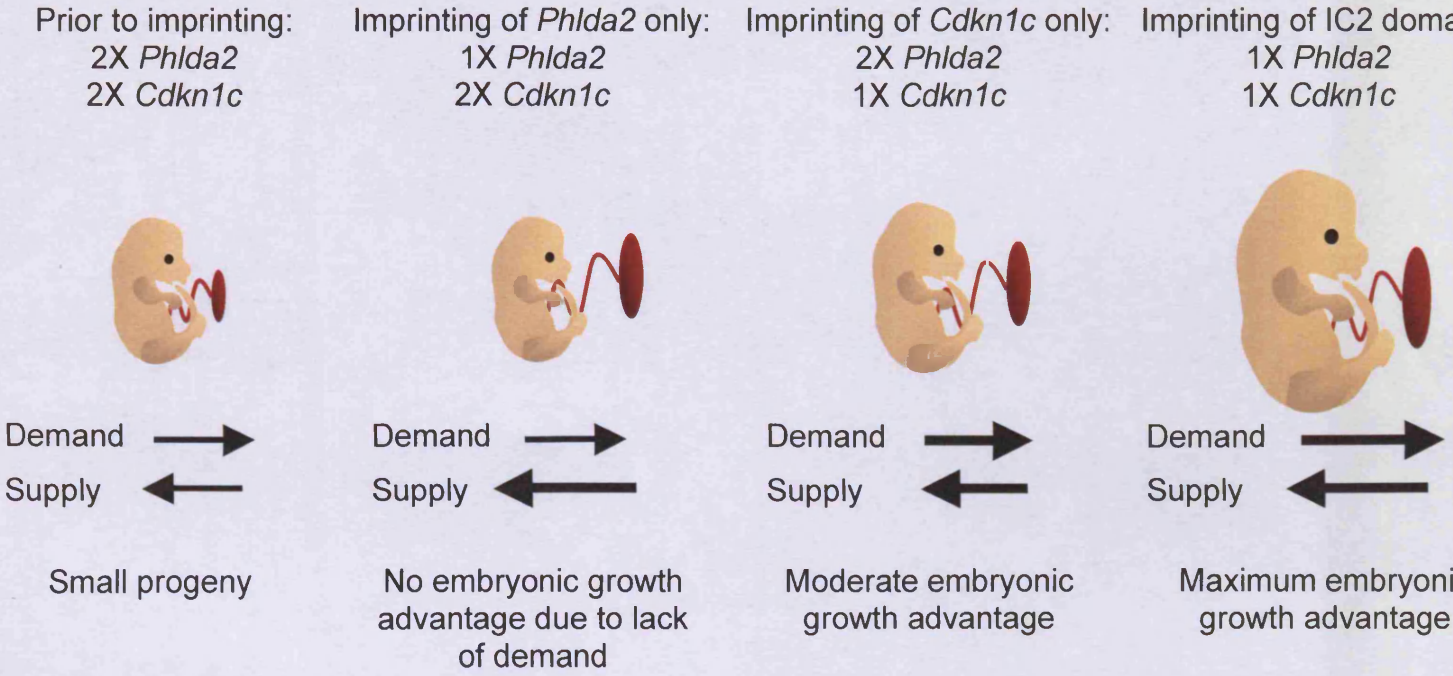


Figure 6.1: Theoretical effects of imprinting *Phlda2* and *Cdkn1c* in isolation and together

The diagram depicts the theoretical growth advantage of imprinting *Phlda2* and *Cdkn1c* in isolation and together. Biallelic expression of the maternally expressed *Phlda2* and *Cdkn1c* was growth restrictive in the ancestral embryo. Monoallelic expression of *Phlda2* in isolation would provide a larger placenta, but in the absence of increased embryonic demand, would impart no growth advantage. Monoallelic expression of *Cdkn1c* would increase the inherent growth potential of the embryo and theoretically increase placental size also, thus resulting in a moderate growth advantage. However, imprinting of both *Phlda2* and *Cdkn1c* would increase embryonic demand and placental supply of nutrients, thus imparting maximum growth advantage to the embryo.

6.1.4 Metabolic syndrome and fetal programming

In addition to identifying the rationale for imprinting of *Phlda2*, the BAC transgenic mice also provided a model to investigate the consequences of loss of imprinting at the discrete locus encompassing *Phlda2* and *Slc22a18*. Imprinting of the IC2 sub-domain is associated with the maternal methylation of *Kvdmr1* and paternal expression of the ncRNA *Kcnq1ot1*, which renders the paternal alleles of genes including *Phlda2*, *Slc22a18* and *Cdkn1c* silent (Smilinich *et al.* 1999). Loss of transcription of the paternal *Kcnq1ot1* transcript results in biallelic expression of the associated genes (Fitzpatrick *et al.* 2002; Mancini-Dinardo *et al.* 2006; Thakur *et al.* 2004). True LOI at distal 7 would therefore be associated with upregulation of genes including *Cdkn1c* and *Ascl2* in addition to *Phlda2* and *Slc22a18*. Nonetheless, the BAC transgenic mice enabled the relative contribution of *Phlda2* and *Scl22a18* to be investigated. Additionally, *PHLDA2* was found to be specifically upregulated in *Dicer* knockdown HEK293 cells as a result of altered chromatin structure with *Cdkn1c* expression remaining monoallelic (Tang *et al.* 2007). This observation suggests that over-expression of *Phlda2* alone is possible *in vitro*.

As discussed previously, the placental defect associated with the BAC transgenic mice can be largely attributed to over-expression of *Phlda2*. The consequence of such over-expression is a significantly lighter placenta from at least E12.5, with embryonic growth adversely affected from E16.5. Transgenic embryos exhibit brain-sparing growth and display rapid post-natal catch up growth. These observations are consistent with the presence of placental insufficiency negatively impacting on embryonic growth. Such individuals are thought to be subject to "fetal programming", such that the embryo adapts to the low-nutrient environment *in utero* and cannot subsequently adapt to a relatively nutrient rich post-natal environment. The consequence of fetal programming is the onset of metabolic syndrome in adulthood, characterised by central obesity, type 2 diabetes, hypertension and cardiovascular disease. The presence of increased adiposity and reduced glucose tolerance in transgenic females at one year of age was indicative of a predisposition to metabolic syndrome, consistent with the placental insufficiency and metabolic programming of transgenic embryos. However, a role for embryonic expression of *Phlda2* and *Slc22a18* in

the observed adult phenotypes cannot be excluded at present. Interestingly, preliminary data indicates that such aspects of metabolic syndrome are absent from transgenic animals of line 5A4. *Phlda2* and *Slc22a18* are over-expressed in line 5A4 similar to line 10-15 but *Cdkn1c* is additionally over-expressed in the embryo, thus intrinsically restricting embryonic growth. This suggests that *Phlda2* and *Slc22a18* do not directly affect adult metabolism. Transgenic animals of line 10-15 exhibit asymmetric growth restriction followed by rapid post-natal catch up growth and an association with increased adiposity and reduced glucose tolerance. In contrast, the additional over-expression of *Cdkn1c* in the embryo of line 5A4 intrinsically restricts embryonic growth, such that post-natal catch up growth is absent and this appears to protect against the onset of metabolic syndrome as summarised in Table 6.1.

In addition to providing further insight into the consequences of imprinting the IC2 domain, these data has important implications for human health. Indeed, elevated *PHLDA2* has been reported in human placentae of IUGR and lower birth weight infants, despite the preserved paternal methylation of *Kvdmr1* (Apostolidou *et al.* 2007; McMinn *et al.* 2006). This data suggests that the ratio of *Phlda2* and *Cdkn1c* expression may be an important diagnostic marker for human IUGR infants. Elevated but balanced *Phlda2* and *Cdkn1c* expression would be associated with reduced embryonic growth but no long-term metabolic consequences. Similarly, an increased *Cdkn1c* to *Phlda2* ratio would indicate an intrinsically small individual with no long-term metabolic consequences. In contrast, an increased *Phlda2* to *Cdkn1c* expression ratio would predict placental insufficiency and asymmetric IUGR, which may be indicative of a predisposition to metabolic syndrome in later life. Elevated *Phlda2* and *Cdkn1c* expression could result from LOI of the domain, although this would result in a balanced over-expression of both *Phlda2* and *Cdkn1c*. However, excess expression of *Phlda2* independent of loss of *Kcnq1ot1* has been demonstrated in a *Dicer* deficient human cell line (Tang *et al.* 2007). Furthermore, maintenance of monoallelic expression of *Cdkn1c* requires additional methylation of the *Cdkn1c* promoter on the paternal chromosome (Bhogal *et al.* 2004). This methylation is dependent upon the chromatin remodelling protein Hells/Lsh (helicase, lymphoid specific), with biallelic expression of *Cdkn1c* observed in Hells deficient mice despite appropriate methylation of *Kvdmr1* (Fan *et al.* 2005). Hells deficient mice

are born approximately ~20% lighter, although they die soon after birth (Geiman *et al.* 2001). Deregulated expression of both *Phlda2* and *Cdkn1c* has thus been demonstrated to occur independent of domain-wide LOI, identifying potential mechanisms through which unbalanced *Phlda2* and *Cdkn1c* expression may result.

One potential complication associated with the BAC transgenic mice utilised in this investigation is the expression of *Phlda2* and *Slc22a18* in a subset of embryonic and adult tissues. The observed adult phenotypes cannot therefore be directly attributed to placental insufficiency, as a direct role for excess expression of these genes in contributing to the adult phenotypes cannot be excluded. However, it is possible that excess *Phlda2* in human placentae is also associated with elevated expression in all tissues in which the gene is endogenously expressed. Whether such expression is directly linked with a predisposition to metabolic syndrome or this is an indirect consequence of placental insufficiency and the associated fetal programming remains unclear. It will be interesting to examine the level of *Phlda2* expression in the kidney of adult human IUGR individuals.

Phlda2	*Cdkn1c*	Consequences
Monoallelic	Monoallelic	Large placenta Intrinsically large embryo Embryonic growth potential is achieved
Biallelic	Monoallelic	Small placenta Intrinsically large embryo Embryonic growth potential is not achieved Asymmetric IUGR Predisposition to metabolic syndrome
Monoallelic	Biallelic	Intermediate placenta Intrinsically small embryo Embryonic growth potential is achieved
Biallelic	Biallelic	Small placenta Intrinsically small embryo Embryonic growth potential is achieved Resistance to metabolic syndrome

Table 6.1: Consequences of unbalanced *Phlda2* and *Cdkn1c* expression

The table summarises the consequences of unbalanced *Phlda2* and *Cdkn1c* expression. Balanced *Phlda2* and *Cdkn1c* expression is not associated with any long-term consequences, with the level of expression associated with embryonic growth potential. Similarly, increased *Cdkn1c* to *Phlda2* expression ratio intrinsically restricts embryonic growth, but has no long-term health consequences. In contrast, an elevated *Phlda2* to *Cdkn1c* ratio would cause placental insufficiency associated with asymmetric IUGR and a predisposition to metabolic syndrome.

6.1.5 Concluding remarks

In summary, over-expression of *Phlda2* in the mouse placenta is associated with a significant reduction in placental growth due to a disproportionate loss and disruption of the spongiotrophoblast layer. Transgenic embryos display asymmetric growth restriction, are born significantly smaller than wild type and exhibit rapid post-natal catch up growth. This placental and embryonic growth pattern is consistent with the presence of placental insufficiency. Additionally, transgenic adult female mice display increased adiposity and reduced glucose tolerance, indicative of a predisposition to metabolic syndrome.

A number of conclusions can be drawn from the data presented in this investigation. Firstly, these data have identified a possible rationale for imprinting *Phlda2*, with reduced expression levels associated with enhanced placental function in order to support the increased embryonic growth potential driven by imprinting of *Cdkn1c*. Secondly, this work has identified *Phlda2* as a potential regulator of the glycogen cell population of the placenta, which represents a novel finding. Thirdly, this work has characterised a progressive and asymmetric embryonic growth restriction phenotype, suggesting a role for deregulated expression of this locus in human IUGR. Finally, there is evidence of an adult metabolic phenotype in transgenic mice of line 10-15. Whether this is due to placental insufficiency or excess expression of *Phlda2* and/or *Slc22a18* in the embryo, or indeed the adult, is currently unknown. Regardless of the cause, this data identifies these mice as a novel genetic model in which both adiposity and glucose tolerance is modified. Further investigations may provide insight into the developmental origins of these potentially life-threatening disorders that are particularly associated with those born with a low birth weight and show catch up growth.

Bibliography

Adair, L. S. 1989. Low birth weight and intrauterine growth retardation in Filipino infants. *Pediatrics* 84(4), pp. 613-622.

Adamson, S. L., Lu, Y., Whiteley, K. J., Holmyard, D., Hemberger, M., Pfarrer, C. and Cross, J. C. 2002. Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Dev Biol* 250(2), pp. 358-373.

Ager, E. I., Pask, A. J., Gehring, H. M., Shaw, G. and Renfree, M. B. 2008. Evolution of the CDKN1C-KCNQ1 imprinted domain. *BMC Evol Biol* 8, p. 163.

Ainscough, J. F., John, R. M. and Surani, M. A. 1998. Mechanism of imprinting on mouse distal chromosome 7. *Genet Res* 72(3), pp. 237-245.

Alberti, K. G., Zimmet, P. and Shaw, J. 2005. The metabolic syndrome--a new worldwide definition. *Lancet* 366(9491), pp. 1059-1062.

Alders, M., Hodges, M., Hadjantonakis, A. K., Postmus, J., van Wijk, I., Bliek, J., de Meulemeester, M., Westerveld, A., Guillemot, F., Oudejans, C., Little, P. and Mannens, M. 1997. The human Achaete-Scute homologue 2 (ASCL2,HASH2) maps to chromosome 11p15.5, close to IGF2 and is expressed in extravillus trophoblasts. *Hum Mol Genet* 6(6), pp. 859-867.

Altshuller, Y., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Frohman, M. A. 1996. Gcm1, a mammalian homolog of Drosophila glial cells missing. *FEBS Lett* 393(2-3), pp. 201-204.

Andrews, S. C., Wood, M. D., Tunster, S. J., Barton, S. C., Surani, M. A. and John, R. M. 2007. Cdkn1c (p57Kip2) is the major regulator of embryonic growth within its imprinted domain on mouse distal chromosome 7. *BMC Dev Biol* 7, p. 53.

Angiolini, E., Fowden, A., Coan, P., Sandovici, I., Smith, P., Dean, W., Burton, G., Tycko, B., Reik, W., Sibley, C. and Constância, M. 2006. Regulation of placental efficiency for nutrient transport by imprinted genes. *Placenta* 27 Suppl, pp. 98-102.

Anson-Cartwright, L., Dawson, K., Holmyard, D., Fisher, S. J., Lazzarini, R. A. and Cross, J. C. 2000. The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. *Nat Genet* 25(3), pp. 311-314.

Antonazzo, P., Alvino, G., Cozzi, V., Grati, F. R., Tabano, S., Sirchia, S., Miozzo, M. and Cetin, I. 2008. Placental IGF2 expression in normal and intrauterine growth restricted (IUGR) pregnancies. *Placenta* 29(1), pp. 99-101.

Apostolidou, S., Abu-Amero, S., O'Donoghue, K., Frost, J., Olafsdottir, O., Chavele, K. M., Whittaker, J. C., Loughna, P., Stanier, P. and Moore, G. E. 2007.

Elevated placental expression of the imprinted PHLDA2 gene is associated with low birth weight. *J Mol Med* 85(4), pp. 379-387.

Arman, E., Haffner-Krausz, R., Chen, Y., Heath, J. K. and Lonai, P. 1998. Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. *Proc Natl Acad Sci U S A* 95(9), pp. 5082-5087.

Ashwell, M., Priest, P., Bondoux, M., Sowter, C. and McPherson, C. K. 1976. Human fat cell sizing--a quick, simple method. *J Lipid Res* 17(2), pp. 190-192.

Ashwell, M. A., Priest, P. and Sowter, C. 1975. Importance of fixed sections in the study of adipose tissue cellularity. *Nature* 256(5520), pp. 724-725.

Avner, P. and Heard, E. 2001. X-chromosome inactivation: counting, choice and initiation. *Nat Rev Genet* 2(1), pp. 59-67.

Baker, J., Liu, J. P., Robertson, E. J. and Efstratiadis, A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75(1), pp. 73-82.

Bamberg, C. and Kalache, K. D. 2004. Prenatal diagnosis of fetal growth restriction. *Semin Fetal Neonatal Med* 9(5), pp. 387-394.

Barker, D. J. 1995. Fetal origins of coronary heart disease. *BMJ* 311(6998), pp. 171-174.

Barker, D. J. 1997. Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition* 13(9), pp. 807-813.

Barker, D. J., Bull, A. R., Osmond, C. and Simmonds, S. J. 1990. Fetal and placental size and risk of hypertension in adult life. *Bmj* 301(6746), pp. 259-262.

Barker, D. J., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A. and Robinson, J. S. 1993. Fetal nutrition and cardiovascular disease in adult life. *Lancet* 341(8850), pp. 938-941.

Barker, D. J. and Osmond, C. 1986. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* 1(8489), pp. 1077-1081.

Barker, M., Robinson, S., Osmond, C. and Barker, D. J. 1997. Birth weight and body fat distribution in adolescent girls. *Arch Dis Child* 77(5), pp. 381-383.

Barlow, D. P. 1993. Methylation and imprinting: from host defense to gene regulation? *Science* 260(5106), pp. 309-310.

Barlow, D. P. 1995. Gametic imprinting in mammals. *Science* 270(5242), pp. 1610-1613.

Barlow, D. P., Stoger, R., Herrmann, B. G., Saito, K. and Schweifer, N. 1991. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature* 349(6304), pp. 84-87.

Barton, S. C., Surani, M. A. and Norris, M. L. 1984. Role of paternal and maternal genomes in mouse development. *Nature* 311(5984), pp. 374-376.

Basyuk, E., Cross, J. C., Corbin, J., Nakayama, H., Hunter, P., Nait-Oumesmar, B. and Lazzarini, R. A. 1999. Murine Gcm1 gene is expressed in a subset of placental trophoblast cells. *Dev Dyn* 214(4), pp. 303-311.

Bell, A. C. and Felsenfeld, G. 2000. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* 405(6785), pp. 482-485.

Bell, A. C., West, A. G. and Felsenfeld, G. 1999. The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell* 98(3), pp. 387-396.

Bestor, T. H. 2000. The DNA methyltransferases of mammals. *Hum Mol Genet* 9(16), pp. 2395-2402.

Bhogal, B., Arnaudo, A., Dymkowski, A., Best, A. and Davis, T. L. 2004. Methylation at mouse Cdkn1c is acquired during postimplantation development and functions to maintain imprinted expression. *Genomics* 84(6), pp. 961-970.

Bieswal, F., Ahn, M. T., Reusens, B., Holvoet, P., Raes, M., Rees, W. D. and Remacle, C. 2006. The importance of catch-up growth after early malnutrition for the programming of obesity in male rat. *Obesity (Silver Spring)* 14(8), pp. 1330-1343.

Bininda-Emonds, O. R., Cardillo, M., Jones, K. E., MacPhee, R. D., Beck, R. M., Grenyer, R., Price, S. A., Vos, R. A., Gittleman, J. L. and Purvis, A. 2007. The delayed rise of present-day mammals. *Nature* 446(7135), pp. 507-512.

Birchler, J. A., Bhadra, M. P. and Bhadra, U. 2000. Making noise about silence: repression of repeated genes in animals. *Curr Opin Genet Dev* 10(2), pp. 211-216.

Bird, A. 1992. The essentials of DNA methylation. *Cell* 70(1), pp. 5-8.

Blair, E. 1994. The myth of fetal growth retardation at term. *Br J Obstet Gynaecol* 101(9), pp. 830-831.

Bouillot, S., Rampon, C., Tillet, E. and Huber, P. 2005. Tracing the Glycogen Cells with Protocadherin 12 During Mouse Placenta Development. *Placenta*.

Branco, M. R., Oda, M. and Reik, W. 2008. Safeguarding parental identity: Dnmt1 maintains imprints during epigenetic reprogramming in early embryogenesis. *Genes Dev* 22(12), pp. 1567-1571.

Breier, G., Clauss, M. and Risau, W. 1995. Coordinate expression of vascular endothelial growth factor receptor-1 (flt-1) and its ligand suggests a paracrine regulation of murine vascular development. *Dev Dyn* 204(3), pp. 228-239.

Brodsky, D. and Christou, H. 2004. Current concepts in intrauterine growth restriction. *J Intensive Care Med* 19(6), pp. 307-319.

Brown, S. W. and Nur, U. 1964. Heterochromatic Chromosomes In The Coccids. *Science* 145, pp. 130-136.

Buck, G. M., Cookfair, D. L., Michalek, A. M., Nasca, P. C., Standfast, S. J., Sever, L. E. and Kramer, A. A. 1989. Intrauterine growth retardation and risk of sudden infant death syndrome (SIDS). *Am J Epidemiol* 129(5), pp. 874-884.

Burdge, G. C., Hanson, M. A., Slater-Jefferies, J. L. and Lillycrop, K. A. 2007. Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *Br J Nutr* 97(6), pp. 1036-1046.

Campbell, S. and Thoms, A. 1977. Ultrasound measurement of the fetal head to abdomen circumference ratio in the assessment of growth retardation. *Br J Obstet Gynaecol* 84(3), pp. 165-174.

Carney, E. W., Prideaux, V., Lye, S. J. and Rossant, J. 1993. Progressive expression of trophoblast-specific genes during formation of mouse trophoblast giant cells in vitro. *Mol Reprod Dev* 34(4), pp. 357-368.

Carter, A. M., Nygard, K., Mazzuca, D. M. and Han, V. K. 2006. The Expression of Insulin-like Growth Factor and Insulin-like Growth Factor Binding Protein mRNAs in Mouse Placenta. *Placenta* 27(2-3), pp. 278-290.

Caspary, T., Cleary, M. A., Baker, C. C., Guan, X. J. and Tilghman, S. M. 1998. Multiple mechanisms regulate imprinting of the mouse distal chromosome 7 gene cluster. *Mol Cell Biol* 18(6), pp. 3466-3474.

Caspary, T., Cleary, M. A., Perlman, E. J., Zhang, P., Elledge, S. J. and Tilghman, S. M. 1999. Oppositely imprinted genes p57(Kip2) and igf2 interact in a mouse model for Beckwith-Wiedemann syndrome. *Genes Dev* 13(23), pp. 3115-3124.

Cattanach, B. M. and Beechey, C. 1997. Genomic imprinting in the mouse: possible final analysis. In: Reik, W. and Surani, A. eds. *Genomic Imprinting*. Oxford University Press, pp. 118-141.

Cattanach, B. M. and Beechey, C. V. 1990. Autosomal and X-chromosome imprinting. *Dev Suppl*, pp. 63-72.

Cattanach, B. M., Beechey, C. V. and Peters, J. 2004. Interactions between imprinting effects in the mouse. *Genetics* 168(1), pp. 397-413.

Cattanach, B. M. and Kirk, M. 1985. Differential activity of maternally and paternally derived chromosome regions in mice. *Nature* 315(6019), pp. 496-498.

Chapman, D. D., Shivji, M. S., Louis, E., Sommer, J., Fletcher, H. and Prodohl, P. A. 2007. Virgin birth in a hammerhead shark. *Biol Lett* 3(4), pp. 425-427.

Charalambous, M., Smith, F. M., Bennett, W. R., Crew, T. E., Mackenzie, F. and Ward, A. 2003. Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism. *Proc Natl Acad Sci U S A* 100(14), pp. 8292-8297.

Chazaud, C., Yamanaka, Y., Pawson, T. and Rossant, J. 2006. Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. *Dev Cell* 10(5), pp. 615-624.

Chess, A., Simon, I., Cedar, H. and Axel, R. 1994. Allelic inactivation regulates olfactory receptor gene expression. *Cell* 78(5), pp. 823-834.

Chevalier-Mariette, C., Henry, I., Montfort, L., Capgras, S., Forlani, S., Muschler, J. and Nicolas, J. F. 2003. CpG content affects gene silencing in mice: evidence from novel transgenes. *Genome Biol* 4(9), p. R53.

Clark, L., Wei, M., Cattoretti, G., Mendelsohn, C. and Tycko, B. 2002. The Tnfrh1 (Tnfrsf23) gene is weakly imprinted in several organs and expressed at the trophoblast-decidua interface. *BMC Genet* 3, p. 11.

Coan, P. M., Burton, G. J. and Ferguson-Smith, A. C. 2005a. Imprinted genes in the placenta--a review. *Placenta* 26 Suppl A, pp. S10-20.

Coan, P. M., Conroy, N., Burton, G. J. and Ferguson-Smith, A. C. 2006. Origin and characteristics of glycogen cells in the developing murine placenta. *Dev Dyn* 235(12), pp. 3280-3294.

Coan, P. M., Ferguson-Smith, A. C. and Burton, G. J. 2004. Developmental dynamics of the definitive mouse placenta assessed by stereology. *Biol Reprod* 70(6), pp. 1806-1813.

Coan, P. M., Ferguson-Smith, A. C. and Burton, G. J. 2005b. Ultrastructural changes in the interhaemal membrane and junctional zone of the murine chorioallantoic placenta across gestation. *J Anat* 207(6), pp. 783-796.

Coan, P. M., Fowden, A. L., Constancia, M., Ferguson-Smith, A. C., Burton, G. J. and Sibley, C. P. 2008. Disproportional effects of Igf2 knockout on placental morphology and diffusional exchange characteristics in the mouse. *J Physiol* 586(Pt 20), pp. 5023-5032.

Constância, M., Angiolini, E., Sandovici, I., Smith, P., Smith, R., Kelsey, G., Dean, W., Ferguson-Smith, A., Sibley, C. P., Reik, W. and Fowden, A. 2005. Adaptation of nutrient supply to fetal demand in the mouse involves interaction

between the Igf2 gene and placental transporter systems. *Proc Natl Acad Sci U S A* 102(52), pp. 19219-19224.

Constância, M., Dean, W., Lopes, S., Moore, T., Kelsey, G. and Reik, W. 2000. Deletion of a silencer element in Igf2 results in loss of imprinting independent of H19. *Nat Genet* 26(2), pp. 203-206.

Constância, M., Hemberger, M., Hughes, J., Dean, W., Ferguson-Smith, A., Fundele, R., Stewart, F., Kelsey, G., Fowden, A., Sibley, C. and Reik, W. 2002. Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature* 417(6892), pp. 945-948.

Constância, M., Pickard, B., Kelsey, G. and Reik, W. 1998. Imprinting mechanisms. *Genome Res* 8(9), pp. 881-900.

Cooper, P. R., Smilinich, N. J., Day, C. D., Nowak, N. J., Reid, L. H., Pearsall, R. S., Reece, M., Prawitt, D., Landers, J., Housman, D. E., Winterpacht, A., Zabel, B. U., Pelletier, J., Weissman, B. E., Shows, T. B. *et al.* 1998. Divergently transcribed overlapping genes expressed in liver and kidney and located in the 11p15.5 imprinted domain. *Genomics* 49(1), pp. 38-51.

Copp, A. J. 1979. Interaction between inner cell mass and trophectoderm of the mouse blastocyst. II. The fate of the polar trophectoderm. *J Embryol Exp Morphol* 51, pp. 109-120.

Corcoran, A. E. 2005. Immunoglobulin locus silencing and allelic exclusion. *Semin Immunol* 17(2), pp. 141-154.

Cross, J. C. 2000. Genetic insights into trophoblast differentiation and placental morphogenesis. *Semin Cell Dev Biol* 11(2), pp. 105-113.

Cross, J. C. 2005. How to make a placenta: mechanisms of trophoblast cell differentiation in mice--a review. *Placenta* 26 Suppl A, pp. S3-9.

Cross, J. C., Baczyk, D., Dobric, N., Hemberger, M., Hughes, M., Simmons, D. G., Yamamoto, H. and Kingdom, J. C. 2003a. Genes, development and evolution of the placenta. *Placenta* 24(2-3), pp. 123-130.

Cross, J. C., Flannery, M. L., Blanar, M. A., Steingrimsson, E., Jenkins, N. A., Copeland, N. G., Rutter, W. J. and Werb, Z. 1995. Hxt encodes a basic helix-loop-helix transcription factor that regulates trophoblast cell development. *Development* 121(8), pp. 2513-2523.

Cross, J. C., Simmons, D. G. and Watson, E. D. 2003b. Chorioallantoic morphogenesis and formation of the placental villous tree. *Ann N Y Acad Sci* 995, pp. 84-93.

Cross, J. C., Werb, Z. and Fisher, S. J. 1994. Implantation and the placenta: key pieces of the development puzzle. *Science* 266(5190), pp. 1508-1518.

Crouse, H. V. 1960. The Controlling Element in Sex Chromosome Behavior in Sciara. *Genetics* 45(10), pp. 1429-1443.

Curley, J. P., Barton, S., Surani, A. and Keverne, E. B. 2004. Coadaptation in mother and infant regulated by a paternally expressed imprinted gene. *Proc Biol Sci* 271(1545), pp. 1303-1309.

Dao, D., Frank, D., Qian, N., O'Keefe, D., Vosatka, R. J., Walsh, C. P. and Tycko, B. 1998. IMPT1, an imprinted gene similar to polyspecific transporter and multi-drug resistance genes. *Hum Mol Genet* 7(4), pp. 597-608.

DeChiara, T. M., Efstratiadis, A. and Robertson, E. J. 1990. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* 345(6270), pp. 78-80.

DeChiara, T. M., Robertson, E. J. and Efstratiadis, A. 1991. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 64(4), pp. 849-859.

Delaval, K. and Feil, R. 2004. Epigenetic regulation of mammalian genomic imprinting. *Curr Opin Genet Dev* 14(2), pp. 188-195.

Desai, M., Crowther, N. J., Lucas, A. and Hales, C. N. 1996. Organ-selective growth in the offspring of protein-restricted mothers. *Br J Nutr* 76(4), pp. 591-603.

Desai, M., Gayle, D., Babu, J. and Ross, M. G. 2005. Programmed obesity in intrauterine growth-restricted newborns: modulation by newborn nutrition. *Am J Physiol Regul Integr Comp Physiol* 288(1), pp. R91-96.

Devriendt, K. 2005. Hydatidiform mole and triploidy: the role of genomic imprinting in placental development. *Hum Reprod Update* 11(2), pp. 137-142.

Dhar, M. S., Sommardahl, C. S., Kirkland, T., Nelson, S., Donnell, R., Johnson, D. K. and Castellani, L. W. 2004. Mice heterozygous for Atp10c, a putative amphipath, represent a novel model of obesity and type 2 diabetes. *J Nutr* 134(4), pp. 799-805.

Dobson, P. C., Abell, D. A. and Beischer, N. A. 1981. Mortality and morbidity of fetal growth retardation. *Aust N Z J Obstet Gynaecol* 21(2), pp. 69-72.

Downs, K. M. and Gardner, R. L. 1995. An investigation into early placental ontogeny: allantoic attachment to the chorion is selective and developmentally regulated. *Development* 121(2), pp. 407-416.

Dumont, D. J., Fong, G. H., Puri, M. C., Gradwohl, G., Alitalo, K. and Breitman, M. L. 1995. Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev Dyn* 203(1), pp. 80-92.

Dunwoodie, S. L. and Beddington, R. S. 2002. The expression of the imprinted gene Ipl is restricted to extra-embryonic tissues and embryonic lateral mesoderm during early mouse development. *Int J Dev Biol* 46(4), pp. 459-466.

Dytham, C. 2003a. The basics. In: Dytham, C. ed. *Choosing and Using Statistics: A Biologists Guide*. Second ed. Blackwell Publishing.

Dytham, C. 2003b. Hypothesis testing, sampling and experimental design. In: Dytham, C. ed. *Choosing and Using Statistics: A Biologists Guide*. Second ed. Blackwell Publishing.

Dytham, C. 2003c. Statistics, variables and distributions. In: Dytham, C. ed. *Choosing and Using Statistics: A Biologists Guide*. Second ed. Blackwell Publishing.

Dytham, C. 2003d. The tests 1: tests to look at differences. In: Dytham, C. ed. *Choosing and Using Statistics: A Biologists Guide*. Second ed. Blackwell Publishing.

Eden, S., Constância, M., Hashimshony, T., Dean, W., Goldstein, B., Johnson, A. C., Keshet, I., Reik, W. and Cedar, H. 2001. An upstream repressor element plays a role in Igf2 imprinting. *Embo J* 20(13), pp. 3518-3525.

Edwards, C. A. and Ferguson-Smith, A. C. 2007. Mechanisms regulating imprinted genes in clusters. *Curr Opin Cell Biol* 19(3), pp. 281-289.

Efstratiadis, A. 1995. Epigenetics. A new whiff of monoallelic expression. *Curr Biol* 5(1), pp. 21-24.

Eggenschwiler, J., Ludwig, T., Fisher, P., Leighton, P. A., Tilghman, S. M. and Efstratiadis, A. 1997. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes Dev* 11(23), pp. 3128-3142.

Engel, N., Thorvaldsen, J. L. and Bartolomei, M. S. 2006. CTCF binding sites promote transcription initiation and prevent DNA methylation on the maternal allele at the imprinted H19/Igf2 locus. *Hum Mol Genet* 15(19), pp. 2945-2954.

Engemann, S., Strodicke, M., Paulsen, M., Franck, O., Reinhardt, R., Lane, N., Reik, W. and Walter, J. 2000. Sequence and functional comparison in the Beckwith-Wiedemann region: implications for a novel imprinting centre and extended imprinting. *Hum Mol Genet* 9(18), pp. 2691-2706.

Eriksson, J., Forsen, T., Tuomilehto, J., Osmond, C. and Barker, D. 2000. Fetal and childhood growth and hypertension in adult life. *Hypertension* 36(5), pp. 790-794.

Eriksson, J. G., Forsen, T., Tuomilehto, J., Winter, P. D., Osmond, C. and Barker, D. J. 1999. Catch-up growth in childhood and death from coronary heart disease: longitudinal study. *Bmj* 318(7181), pp. 427-431.

Falls, J. G., Pulford, D. J., Wylie, A. A. and Jirtle, R. L. 1999. Genomic imprinting: implications for human disease. *Am J Pathol* 154(3), pp. 635-647.

Fan, T., Hagan, J. P., Kozlov, S. V., Stewart, C. L. and Muegge, K. 2005. Lsh controls silencing of the imprinted Cdkn1c gene. *Development* 132(4), pp. 635-644.

Fay, R. A. and Ellwood, D. 1993. Categories of intrauterine growth retardation. *Fetal and Maternal Medicine Review* 5, pp. 203-212.

Feil, R. and Berger, F. 2007. Convergent evolution of genomic imprinting in plants and mammals. *Trends Genet* 23(4), pp. 192-199.

Feil, R., Walter, J., Allen, N. D. and Reik, W. 1994. Developmental control of allelic methylation in the imprinted mouse Igf2 and H19 genes. *Development* 120(10), pp. 2933-2943.

Feldman, B., Poueymirou, W., Papaioannou, V. E., DeChiara, T. M. and Goldfarb, M. 1995. Requirement of FGF-4 for postimplantation mouse development. *Science* 267(5195), pp. 246-249.

Ferguson-Smith, A. C., Cattanach, B. M., Barton, S. C., Beechey, C. V. and Surani, M. A. 1991. Embryological and molecular investigations of parental imprinting on mouse chromosome 7. *Nature* 351(6328), pp. 667-670.

Ferguson-Smith, A. C., Sasaki, H., Cattanach, B. M. and Surani, M. A. 1993. Parental-origin-specific epigenetic modification of the mouse H19 gene. *Nature* 362(6422), pp. 751-755.

Ferguson-Smith, A. C. and Surani, M. A. 2001. Imprinting and the epigenetic asymmetry between parental genomes. *Science* 293(5532), pp. 1086-1089.

Fernandez-Twinn, D. S., Wayman, A., Ekizoglou, S., Martin, M. S., Hales, C. N. and Ozanne, S. E. 2005. Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. *Am J Physiol Regul Integr Comp Physiol* 288(2), pp. R368-373.

Fisher, A. M., Thomas, N. S., Cockwell, A., Stecko, O., Kerr, B., Temple, I. K. and Clayton, P. 2002. Duplications of chromosome 11p15 of maternal origin result in a phenotype that includes growth retardation. *Hum Genet* 111(3), pp. 290-296.

Fitzpatrick, G. V., Soloway, P. D. and Higgins, M. J. 2002. Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. *Nat Genet* 32(3), pp. 426-431.

Font de Mora, J., Esteban, L. M., Burks, D. J., Nunez, A., Garces, C., Garcia-Barrado, M. J., Iglesias-Osma, M. C., Moratinos, J., Ward, J. M. and Santos, E. 2003. Ras-GRF1 signaling is required for normal beta-cell development and glucose homeostasis. *Embo J* 22(12), pp. 3039-3049.

Forsen, T., Eriksson, J., Tuomilehto, J., Reunanen, A., Osmond, C. and Barker, D. 2000. The fetal and childhood growth of persons who develop type 2 diabetes. *Ann Intern Med* 133(3), pp. 176-182.

Forsen, T., Eriksson, J. G., Tuomilehto, J., Osmond, C. and Barker, D. J. 1999. Growth in utero and during childhood among women who develop coronary heart disease: longitudinal study. *Bmj* 319(7222), pp. 1403-1407.

Fowden, A. L. 2003. The insulin-like growth factors and feto-placental growth. *Placenta* 24(8-9), pp. 803-812.

Frank, D., Fortino, W., Clark, L., Musalo, R., Wang, W., Saxena, A., Li, C. M., Reik, W., Ludwig, T. and Tycko, B. 2002. Placental overgrowth in mice lacking the imprinted gene Ipl. *Proc Natl Acad Sci U S A* 99(11), pp. 7490-7495.

Frank, D., Mendelsohn, C. L., Ciccone, E., Svensson, K., Ohlsson, R. and Tycko, B. 1999. A novel pleckstrin homology-related gene family defined by Ipl/Tssc3, TDAG51, and Tih1: tissue-specific expression, chromosomal location, and parental imprinting. *Mamm Genome* 10(12), pp. 1150-1159.

Frederich, R. C., Hamann, A., Anderson, S., Lollmann, B., Lowell, B. B. and Flier, J. S. 1995. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat Med* 1(12), pp. 1311-1314.

Gale, C. R., Martyn, C. N., Kellingray, S., Eastell, R. and Cooper, C. 2001. Intrauterine programming of adult body composition. *J Clin Endocrinol Metab* 86(1), pp. 267-272.

Geiman, T. M., Tessarollo, L., Anver, M. R., Kopp, J. B., Ward, J. M. and Muegge, K. 2001. Lsh, a SNF2 family member, is required for normal murine development. *Biochim Biophys Acta* 1526(2), pp. 211-220.

Georgiades, P., Ferguson-Smith, A. C. and Burton, G. J. 2002. Comparative developmental anatomy of the murine and human definitive placentae. *Placenta* 23(1), pp. 3-19.

Georgiades, P., Watkins, M., Burton, G. J. and Ferguson-Smith, A. C. 2001. Roles for genomic imprinting and the zygotic genome in placental development. *Proc Natl Acad Sci U S A* 98(8), pp. 4522-4527.

Georgiades, P., Watkins, M., Surani, M. A. and Ferguson-Smith, A. C. 2000. Parental origin-specific developmental defects in mice with uniparental disomy for chromosome 12. *Development* 127(21), pp. 4719-4728.

Gicquel, C., Rossignol, S., Cabrol, S., Houang, M., Steunou, V., Barbu, V., Danton, F., Thibaud, N., Le Merrer, M., Burglen, L., Bertrand, A. M., Netchine, I. and Le Bouc, Y. 2005. Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. *Nat Genet* 37(9), pp. 1003-1007.

Gilbert, S. F. 2003. *Early Embryonic Development* ***from*** *Developmental Biology*. 7th ed. Sunderland, Massachusetts: Sinauer Associates, Inc.

Glenn, C. C., Driscoll, D. J., Yang, T. P. and Nicholls, R. D. 1997. Genomic imprinting: potential function and mechanisms revealed by the Prader-Willi and Angelman syndromes. *Mol Hum Reprod* 3(4), pp. 321-332.

Gluckman, P. D., Hanson, M. A. and Beedle, A. S. 2007. Non-genomic transgenerational inheritance of disease risk. *Bioessays* 29(2), pp. 145-154.

Gnarra, J. R., Ward, J. M., Porter, F. D., Wagner, J. R., Devor, D. E., Grinberg, A., Emmert-Buck, M. R., Westphal, H., Klausner, R. D. and Linehan, W. M. 1997. Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. *Proc Natl Acad Sci U S A* 94(17), pp. 9102-9107.

Godfrey, K. M., Lillycrop, K. A., Burdge, G. C., Gluckman, P. D. and Hanson, M. A. 2007. Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease. *Pediatr Res* 61(5 Pt 2), pp. 5R-10R.

Goldmit, M. and Bergman, Y. 2004. Monoallelic gene expression: a repertoire of recurrent themes. *Immunol Rev* 200, pp. 197-214.

Graham, C. F. 1974. The production of parthenogenetic mammalian embryos and their use in biological research. *Biol Rev Camb Philos Soc* 49(3), pp. 399-424.

Guillemot, F., Caspary, T., Tilghman, S. M., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Anderson, D. J., Joyner, A. L., Rossant, J. and Nagy, A. 1995. Genomic imprinting of Mash2, a mouse gene required for trophoblast development. *Nat Genet* 9(3), pp. 235-242.

Guillemot, F., Nagy, A., Auerbach, A., Rossant, J. and Joyner, A. L. 1994. Essential role of Mash-2 in extraembryonic development. *Nature* 371(6495), pp. 333-336.

Hadchouel, M., Farza, H., Simon, D., Tiollais, P. and Pourcel, C. 1987. Maternal inhibition of hepatitis B surface antigen gene expression in transgenic mice correlates with de novo methylation. *Nature* 329(6138), pp. 454-456.

Haffner-Krausz, R., Gorivodsky, M., Chen, Y. and Lonai, P. 1999. Expression of Fgfr2 in the early mouse embryo indicates its involvement in preimplantation development. *Mech Dev* 85(1-2), pp. 167-172.

Hagan, J. P., Kozlov, S. V., Chiang, Y., Sewell, L. and Stewart, C. L. 2004. Intraspecific mating with CzechII/Ei mice rescue lethality associated with loss of function mutations of the imprinted genes, Igf2r and Cdkn1c. *Genomics* 84(5), pp. 836-843.

Haig, D. 1994. Refusing the ovarian time bomb. *Trends Genet* 10(10), pp. 346-347; author reply 348-349.

Haig, D. and Graham, C. 1991. Genomic imprinting and the strange case of the insulin-like growth factor II receptor. *Cell* 64(6), pp. 1045-1046.

Hales, C. N. and Barker, D. J. 1992. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* 35(7), pp. 595-601.

Hales, C. N. and Barker, D. J. 2001. The thrifty phenotype hypothesis. *Br Med Bull* 60, pp. 5-20.

Hales, C. N., Barker, D. J., Clark, P. M., Cox, L. J., Fall, C., Osmond, C. and Winter, P. D. 1991. Fetal and infant growth and impaired glucose tolerance at age 64. *Bmj* 303(6809), pp. 1019-1022.

Hall, J. G. 1990. Genomic imprinting: review and relevance to human diseases. *Am J Hum Genet* 46(5), pp. 857-873.

Hart, A. H., Hartley, L., Ibrahim, M. and Robb, L. 2004. Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. *Dev Dyn* 230(1), pp. 187-198.

Haslam, R. J., Koide, H. B. and Hemmings, B. A. 1993. Pleckstrin domain homology. *Nature* 363(6427), pp. 309-310.

Hata, K., Okano, M., Lei, H. and Li, E. 2002. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development* 129(8), pp. 1983-1993.

Hatada, I. and Mukai, T. 1995. Genomic imprinting of p57KIP2, a cyclin-dependent kinase inhibitor, in mouse. *Nat Genet* 11(2), pp. 204-206.

Hatada, I., Ohashi, H., Fukushima, Y., Kaneko, Y., Inoue, M., Komoto, Y., Okada, A., Ohishi, S., Nabetani, A., Morisaki, H., Nakayama, M., Niikawa, N. and Mukai, T. 1996. An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome. *Nat Genet* 14(2), pp. 171-173.

Hediger, M. L., Overpeck, M. D., Kuczmarski, R. J., McGlynn, A., Maurer, K. R. and Davis, W. W. 1998. Muscularity and fatness of infants and young children born small- or large-for-gestational-age. *Pediatrics* 102(5), p. E60.

Hemberger, M. 2008. IFPA award in placentology lecture - characteristics and significance of trophoblast giant cells. *Placenta* 29 Suppl A, pp. S4-9.

Hemberger, M., Nozaki, T., Winterhager, E., Yamamoto, H., Nakagama, H., Kamada, N., Suzuki, H., Ohta, T., Ohki, M., Masutani, M. and Cross, J. C. 2003. Parp1-deficiency induces differentiation of ES cells into trophoblast derivatives. *Dev Biol* 257(2), pp. 371-381.

Henriksen, T. and Clausen, T. 2002. The fetal origins hypothesis: placental insufficiency and inheritance versus maternal malnutrition in well-nourished populations. *Acta Obstet Gynecol Scand* 81(2), pp. 112-114.

Henry, I., Bonaiti-Pellie, C., Chehensse, V., Beldjord, C., Schwartz, C., Utermann, G. and Junien, C. 1991. Uniparental paternal disomy in a genetic cancer-predisposing syndrome. *Nature* 351(6328), pp. 665-667.

Hirasawa, R., Chiba, H., Kaneda, M., Tajima, S., Li, E., Jaenisch, R. and Sasaki, H. 2008. Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. *Genes Dev* 22(12), pp. 1607-1616.

Hitz, C., Vogt-Weisenhorn, D., Ruiz, P., Wurst, W. and Floss, T. 2005. Progressive loss of the spongiotrophoblast layer of Birc6/Bruce mutants results in embryonic lethality. *Genesis* 42(2), pp. 91-103.

Hokken-Koelega, A. C., De Ridder, M. A., Lemmen, R. J., Den Hartog, H., De Muinck Keizer-Schrama, S. M. and Drop, S. L. 1995. Children born small for gestational age: do they catch up? *Pediatr Res* 38(2), pp. 267-271.

Hu, J. F., Oruganti, H., Vu, T. H. and Hoffman, A. R. 1998. Tissue-specific imprinting of the mouse insulin-like growth factor II receptor gene correlates with differential allele-specific DNA methylation. *Mol Endocrinol* 12(2), pp. 220-232.

Hu, R. J., Lee, M. P., Johnson, L. A. and Feinberg, A. P. 1996. A novel human homologue of yeast nucleosome assembly protein, 65 kb centromeric to the p57KIP2 gene, is biallelically expressed in fetal and adult tissues. *Hum Mol Genet* 5(11), pp. 1743-1748.

Hurst, L. 1997. *Evolutionary theories of genomic imprinting.*

Hurst, L. D. and McVean, G. T. 1998. Do we understand the evolution of genomic imprinting? *Curr Opin Genet Dev* 8(6), pp. 701-708.

Inoue, K., Kohda, T., Lee, J., Ogonuki, N., Mochida, K., Noguchi, Y., Tanemura, K., Kaneko-Ishino, T., Ishino, F. and Ogura, A. 2002. Faithful expression of imprinted genes in cloned mice. *Science* 295(5553), p. 297.

Isles, A. R. and Holland, A. J. 2005. Imprinted genes and mother-offspring interactions. *Early Hum Dev* 81(1), pp. 73-77.

Jay, P., Rougeulle, C., Massacrier, A., Moncla, A., Mattei, M. G., Malzac, P., Roeckel, N., Taviaux, S., Lefranc, J. L., Cau, P., Berta, P., Lalande, M. and Muscatelli, F. 1997. The human necdin gene, NDN, is maternally imprinted and located in the Prader-Willi syndrome chromosomal region. *Nat Genet* 17(3), pp. 357-361.

Jelinic, P. and Shaw, P. 2007. Loss of imprinting and cancer. *J Pathol* 211(3), pp. 261-268.

Jimenez-Chillaron, J. C., Hernandez-Valencia, M., Lightner, A., Faucette, R. R., Reamer, C., Przybyla, R., Ruest, S., Barry, K., Otis, J. P. and Patti, M. E. 2006. Reductions in caloric intake and early postnatal growth prevent glucose intolerance and obesity associated with low birthweight. *Diabetologia* 49(8), pp. 1974-1984.

Jimenez-Chillaron, J. C., Hernandez-Valencia, M., Reamer, C., Fisher, S., Joszi, A., Hirshman, M., Oge, A., Walrond, S., Przybyla, R., Boozer, C., Goodyear, L. J. and Patti, M. E. 2005. Beta-cell secretory dysfunction in the pathogenesis of low birth weight-associated diabetes: a murine model. *Diabetes* 54(3), pp. 702-711.

Jimenez-Chillaron, J. C. and Patti, M. E. 2007. To catch up or not to catch up: is this the question? Lessons from animal models. *Curr Opin Endocrinol Diabetes Obes* 14(1), pp. 23-29.

John, R. M., Ainscough, J. F., Barton, S. C. and Surani, M. A. 2001. Distant cis-elements regulate imprinted expression of the mouse p57(Kip2) (Cdkn1c) gene: implications for the human disorder, Beckwith--Wiedemann syndrome. *Hum Mol Genet* 10(15), pp. 1601-1609.

John, R. M. and Surani, M. A. 2000. Genomic imprinting, mammalian evolution, and the mystery of egg-laying mammals. *Cell* 101(6), pp. 585-588.

Jollie, W. P. 1990. Development, morphology, and function of the yolk-sac placenta of laboratory rodents. *Teratology* 41(4), pp. 361-381.

Kagitani, F., Kuroiwa, Y., Wakana, S., Shiroishi, T., Miyoshi, N., Kobayashi, S., Nishida, M., Kohda, T., Kaneko-Ishino, T. and Ishino, F. 1997. Peg5/Neuronatin is an imprinted gene located on sub-distal chromosome 2 in the mouse. *Nucleic Acids Res* 25(17), pp. 3428-3432.

Kajii, T. and Ohama, K. 1977. Androgenetic origin of hydatidiform mole. *Nature* 268(5621), pp. 633-634.

Kanduri, C., Pant, V., Loukinov, D., Pugacheva, E., Qi, C. F., Wolffe, A., Ohlsson, R. and Lobanenkov, V. V. 2000. Functional association of CTCF with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive. *Curr Biol* 10(14), pp. 853-856.

Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E. and Sasaki, H. 2004. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 429(6994), pp. 900-903.

Kaneko-Ishino, T., Kohda, T. and Ishino, F. 2003. The regulation and biological significance of genomic imprinting in mammals. *J Biochem (Tokyo)* 133(6), pp. 699-711.

Kaneko-Ishino, T., Kuroiwa, Y., Miyoshi, N., Kohda, T., Suzuki, R., Yokoyama, M., Viville, S., Barton, S. C., Ishino, F. and Surani, M. A. 1995. Peg1/Mest

imprinted gene on chromosome 6 identified by cDNA subtraction hybridization. *Nat Genet* 11(1), pp. 52-59.

Kato, Y., Kaneda, M., Hata, K., Kumaki, K., Hisano, M., Kohara, Y., Okano, M., Li, E., Nozaki, M. and Sasaki, H. 2007. Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. *Hum Mol Genet* 16(19), pp. 2272-2280.

Kaufman, M. H., Barton, S. C. and Surani, M. A. 1977. Normal postimplantation development of mouse parthenogenetic embryos to the forelimb bud stage. *Nature* 265(5589), pp. 53-55.

Kermicle, J. L. 1970. Dependence of the R-Mottled Aleurone Phenotype in Maize on Mode of Sexual Transmission. *Genetics* 66(1), pp. 69-85.

Khatib, H. 2007. Is it genomic imprinting or preferential expression? *Bioessays* 29(10), pp. 1022-1028.

Khatib, H., Zaitoun, I. and Kim, E. S. 2007. Comparative analysis of sequence characteristics of imprinted genes in human, mouse, and cattle. *Mamm Genome* 18(6-7), pp. 538-547.

Killian, J. K., Buckley, T. R., Stewart, N., Munday, B. L. and Jirtle, R. L. 2001. Marsupials and Eutherians reunited: genetic evidence for the Theria hypothesis of mammalian evolution. *Mamm Genome* 12(7), pp. 513-517.

Killian, J. K., Byrd, J. C., Jirtle, J. V., Munday, B. L., Stoskopf, M. K., MacDonald, R. G. and Jirtle, R. L. 2000. M6P/IGF2R imprinting evolution in mammals. *Mol Cell* 5(4), pp. 707-716.

Kim, H. S., Roh, C. R., Chen, B., Tycko, B., Nelson, D. M. and Sadovsky, Y. 2007. Hypoxia regulates the expression of PHLDA2 in primary term human trophoblasts. *Placenta* 28(2-3), pp. 77-84.

Kingdom, J., Huppertz, B., Seaward, G. and Kaufmann, P. 2000. Development of the placental villous tree and its consequences for fetal growth. *Eur J Obstet Gynecol Reprod Biol* 92(1), pp. 35-43.

Kinoshita, T., Yadegari, R., Harada, J. J., Goldberg, R. B. and Fischer, R. L. 1999. Imprinting of the MEDEA polycomb gene in the Arabidopsis endosperm. *Plant Cell* 11(10), pp. 1945-1952.

Kishino, T., Lalande, M. and Wagstaff, J. 1997. UBE3A/E6-AP mutations cause Angelman syndrome. *Nat Genet* 15(1), pp. 70-73.

Knoll, J. H., Nicholls, R. D., Magenis, R. E., Graham, J. M., Jr., Lalande, M. and Latt, S. A. 1989. Angelman and Prader-Willi syndromes share a common chromosome 15 deletion but differ in parental origin of the deletion. *Am J Med Genet* 32(2), pp. 285-290.

Koch, C. A. and Platt, J. L. 2007. T cell recognition and immunity in the fetus and mother. *Cell Immunol* 248(1), pp. 12-17.

Kok, J. H., den Ouden, A. L., Verloove-Vanhorick, S. P. and Brand, R. 1998. Outcome of very preterm small for gestational age infants: the first nine years of life. *Br J Obstet Gynaecol* 105(2), pp. 162-168.

Kono, T. 2006. Genomic imprinting is a barrier to parthenogenesis in mammals. *Cytogenet Genome Res* 113(1-4), pp. 31-35.

Kono, T., Obata, Y., Wu, Q., Niwa, K., Ono, Y., Yamamoto, Y., Park, E. S., Seo, J. S. and Ogawa, H. 2004. Birth of parthenogenetic mice that can develop to adulthood. *Nature* 428(6985), pp. 860-864.

Kono, T., Sotomaru, Y., Katsuzawa, Y. and Dandolo, L. 2002. Mouse parthenogenetic embryos with monoallelic H19 expression can develop to day 17.5 of gestation. *Dev Biol* 243(2), pp. 294-300.

Kraut, N., Snider, L., Chen, C. M., Tapscott, S. J. and Groudine, M. 1998. Requirement of the mouse I-mfa gene for placental development and skeletal patterning. *Embo J* 17(21), pp. 6276-6288.

Kromer, B., Finkenzeller, D., Wessels, J., Dveksler, G., Thompson, J. and Zimmermann, W. 1996. Coordinate expression of splice variants of the murine pregnancy-specific glycoprotein (PSG) gene family during placental development. *Eur J Biochem* 242(2), pp. 280-287.

Kunath, T., Strumpf, D. and Rossant, J. 2004. Early trophoblast determination and stem cell maintenance in the mouse--a review. *Placenta* 25 Suppl A, pp. S32-38.

Lalande, M. 1997. Parental imprinting and human disease. *Annu Rev Genet* 30, pp. 173-195.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J. *et al.* 2001. Initial sequencing and analysis of the human genome. *Nature* 409(6822), pp. 860-921.

Lau, M. M., Stewart, C. E., Liu, Z., Bhatt, H., Rotwein, P. and Stewart, C. L. 1994. Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev* 8(24), pp. 2953-2963.

Law, C. M., Barker, D. J., Osmond, C., Fall, C. H. and Simmonds, S. J. 1992. Early growth and abdominal fatness in adult life. *J Epidemiol Community Health* 46(3), pp. 184-186.

Law, C. M., de Swiet, M., Osmond, C., Fayers, P. M., Barker, D. J., Cruddas, A. M. and Fall, C. H. 1993. Initiation of hypertension in utero and its amplification throughout life. *BMJ* 306(6869), pp. 24-27.

Lawler, S. D., Povey, S., Fisher, R. A. and Pickthall, V. J. 1982. Genetic studies on hydatidiform moles. II. The origin of complete moles. *Ann Hum Genet* 46(Pt 3), pp. 209-222.

Lawson, K. A. and Pedersen, R. A. 1987. Cell fate, morphogenetic movement and population kinetics of embryonic endoderm at the time of germ layer formation in the mouse. *Development* 101(3), pp. 627-652.

Lee, M. H., Reynisdottir, I. and Massague, J. 1995. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev* 9(6), pp. 639-649.

Lee, M. P., Brandenburg, S., Landes, G. M., Adams, M., Miller, G. and Feinberg, A. P. 1999a. Two novel genes in the center of the 11p15 imprinted domain escape genomic imprinting. *Hum Mol Genet* 8(4), pp. 683-690.

Lee, M. P., DeBaun, M. R., Mitsuya, K., Galonek, H. L., Brandenburg, S., Oshimura, M. and Feinberg, A. P. 1999b. Loss of imprinting of a paternally expressed transcript, with antisense orientation to KVLQT1, occurs frequently in Beckwith-Wiedemann syndrome and is independent of insulin-like growth factor II imprinting. *Proc Natl Acad Sci U S A* 96(9), pp. 5203-5208.

Lefebvre, L., Viville, S., Barton, S. C., Ishino, F., Keverne, E. B. and Surani, M. A. 1998. Abnormal maternal behaviour and growth retardation associated with loss of the imprinted gene Mest. *Nat Genet* 20(2), pp. 163-169.

Leger, J., Limoni, C. and Czernichow, P. 1997. Prediction of the outcome of growth at 2 years of age in neonates with intra-uterine growth retardation. *Early Hum Dev* 48(3), pp. 211-223.

Leighton, P. A., Ingram, R. S., Eggenschwiler, J., Efstratiadis, A. and Tilghman, S. M. 1995a. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature* 375(6526), pp. 34-39.

Leighton, P. A., Saam, J. R., Ingram, R. S., Stewart, C. L. and Tilghman, S. M. 1995b. An enhancer deletion affects both H19 and Igf2 expression. *Genes Dev* 9(17), pp. 2079-2089.

Leiter, E. H. 1989. The genetics of diabetes susceptibility in mice. *FASEB J* 3(11), pp. 2231-2241.

Lemmon, M. A. and Ferguson, K. M. 2000. Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochem J* 350 Pt 1, pp. 1-18.

Lescisin, K. R., Varmuza, S. and Rossant, J. 1988. Isolation and characterization of a novel trophoblast-specific cDNA in the mouse. *Genes Dev* 2(12A), pp. 1639-1646.

Lewis, A. and Reik, W. 2006. How imprinting centres work. *Cytogenet Genome Res* 113(1-4), pp. 81-89.

Li, E., Beard, C. and Jaenisch, R. 1993. Role for DNA methylation in genomic imprinting. *Nature* 366(6453), pp. 362-365.

Li, Y. and Behringer, R. R. 1998. Esx1 is an X-chromosome-imprinted regulator of placental development and fetal growth. *Nat Genet* 20(3), pp. 309-311.

Lin, C. C. and Santolaya-Forgas, J. 1998. Current concepts of fetal growth restriction: part I. Causes, classification, and pathophysiology. *Obstet Gynecol* 92(6), pp. 1044-1055.

Lin, C. C., Su, S. J. and River, L. P. 1991. Comparison of associated high-risk factors and perinatal outcome between symmetric and asymmetric fetal intrauterine growth retardation. *Am J Obstet Gynecol* 164(6 Pt 1), pp. 1535-1541; discussion 1541-1532.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4), pp. 402-408.

Loos, R. J., Beunen, G., Fagard, R., Derom, C. and Vlietinck, R. 2001. Birth weight and body composition in young adult men--a prospective twin study. *Int J Obes Relat Metab Disord* 25(10), pp. 1537-1545.

Lopez, M. F., Dikkes, P., Zurakowski, D. and Villa-Komaroff, L. 1996. Insulin-like growth factor II affects the appearance and glycogen content of glycogen cells in the murine placenta. *Endocrinology* 137(5), pp. 2100-2108.

Lubchenco, L. O., Hansman, C., Dressler, M. and Boyd, E. 1963. Intrauterine Growth As Estimated From Liveborn Birth-Weight Data At 24 To 42 Weeks Of Gestation. *Pediatrics* 32, pp. 793-800.

Luedi, P. P., Hartemink, A. J. and Jirtle, R. L. 2005. Genome-wide prediction of imprinted murine genes. *Genome Res* 15(6), pp. 875-884.

Lumbers, E. R., Yu, Z. Y. and Gibson, K. J. 2001. The selfish brain and the barker hypothesis. *Clin Exp Pharmacol Physiol* 28(11), pp. 942-947.

Lyon, M. F. 1961. Gene action in the X-chromosome of the mouse (Mus musculus L.). *Nature* 190, pp. 372-373.

Ma, D., Shield, J. P., Dean, W., Leclerc, I., Knauf, C., Burcelin, R. R., Rutter, G. A. and Kelsey, G. 2004. Impaired glucose homeostasis in transgenic mice

expressing the human transient neonatal diabetes mellitus locus, TNDM. *J Clin Invest* 114(3), pp. 339-348.

Maeda, N. and Hayashizaki, Y. 2006. Genome-wide survey of imprinted genes. *Cytogenet Genome Res* 113(1-4), pp. 144-152.

Malassine, A., Frendo, J. L. and Evain-Brion, D. 2003. A comparison of placental development and endocrine functions between the human and mouse model. *Hum Reprod Update* 9(6), pp. 531-539.

Mancini-Dinardo, D., Steele, S. J., Levorse, J. M., Ingram, R. S. and Tilghman, S. M. 2006. Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes. *Genes Dev* 20(10), pp. 1268-1282.

Mandruzzato, G., Antsaklis, A., Botet, F., Chervenak, F. A., Figueras, F., Grunebaum, A., Puerto, B., Skupski, D. and Stanojevic, M. 2008. Intrauterine restriction (IUGR). *J Perinat Med* 36(4), pp. 277-281.

Mann, J. R. 2001. Imprinting in the germ line. *Stem Cells* 19(4), pp. 287-294.

Mann, J. R. and Lovell-Badge, R. H. 1984. Inviability of parthenogenones is determined by pronuclei, not egg cytoplasm. *Nature* 310(5972), pp. 66-67.

Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W. and Elledge, S. J. 1995. p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev* 9(6), pp. 650-662.

McGrath, J. and Solter, D. 1984. Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* 37(1), pp. 179-183.

McIntire, D. D., Bloom, S. L., Casey, B. M. and Leveno, K. J. 1999. Birth weight in relation to morbidity and mortality among newborn infants. *N Engl J Med* 340(16), pp. 1234-1238.

McLaren, A. 1965. Genetic and Environmental Effects on Foetal and Placental Growth in Mice. *J Reprod Fertil* 9, pp. 79-98.

McLaughlin, K. J., Szabo, P., Haegel, H. and Mann, J. R. 1996. Mouse embryos with paternal duplication of an imprinted chromosome 7 region die at midgestation and lack placental spongiotrophoblast. *Development* 122(1), pp. 265-270.

McMillen, I. C. and Robinson, J. S. 2005. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol Rev* 85(2), pp. 571-633.

McMinn, J., Wei, M., Schupf, N., Cusmai, J., Johnson, E. B., Smith, A. C., Weksberg, R., Thaker, H. M. and Tycko, B. 2006. Unbalanced placental

expression of imprinted genes in human intrauterine growth restriction. *Placenta* 27(6-7), pp. 540-549.

McNeely, M. J., Fujimoto, W. Y., Leonetti, D. L., Tsai, E. C. and Boyko, E. J. 2007. The association between birth weight and visceral fat in middle-age adults. *Obesity (Silver Spring)* 15(4), pp. 816-819.

Miyamoto, T., Hasuike, S., Jinno, Y., Soejima, H., Yun, K., Miura, K., Ishikawa, M. and Niikawa, N. 2002. The human ASCL2 gene escaping genomic imprinting and its expression pattern. *J Assist Reprod Genet* 19(5), pp. 240-244.

Miyoshi, N., Kuroiwa, Y., Kohda, T., Shitara, H., Yonekawa, H., Kawabe, T., Hasegawa, H., Barton, S. C., Surani, M. A., Kaneko-Ishino, T. and Ishino, F. 1998. Identification of the Meg1/Grb10 imprinted gene on mouse proximal chromosome 11, a candidate for the Silver-Russell syndrome gene. *Proc Natl Acad Sci U S A* 95(3), pp. 1102-1107.

Mochizuki, A., Takeda, Y. and Iwasa, Y. 1996. The evolution of genomic imprinting. *Genetics* 144(3), pp. 1283-1295.

Monk, D., Arnaud, P., Apostolidou, S., Hills, F. A., Kelsey, G., Stanier, P., Feil, R. and Moore, G. E. 2006. Limited evolutionary conservation of imprinting in the human placenta. *Proc Natl Acad Sci U S A* 103(17), pp. 6623-6628.

Monk, D. and Moore, G. E. 2004. Intrauterine growth restriction--genetic causes and consequences. *Semin Fetal Neonatal Med* 9(5), pp. 371-378.

Montoliu, L., Chavez, S. and Vidal, M. 2000. Variegation associated with lacZ in transgenic animals: a warning note. *Transgenic Res* 9(3), pp. 237-239.

Moore, T. 1994. Refusing the ovarian time bomb. *Trends Genet* 10(10), pp. 347-349.

Moore, T. 2001. Genetic conflict, genomic imprinting and establishment of the epigenotype in relation to growth. *Reproduction* 122(2), pp. 185-193.

Moore, T., Constância, M., Zubair, M., Bailleul, B., Feil, R., Sasaki, H. and Reik, W. 1997. Multiple imprinted sense and antisense transcripts, differential methylation and tandem repeats in a putative imprinting control region upstream of mouse Igf2. *Proc Natl Acad Sci U S A* 94(23), pp. 12509-12514.

Moore, T. and Haig, D. 1991. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet* 7(2), pp. 45-49.

Morison, I. M., Ramsay, J. P. and Spencer, H. G. 2005. A census of mammalian imprinting. *Trends Genet* 21(8), pp. 457-465.

Morison, I. M. and Reeve, A. E. 1998. A catalogue of imprinted genes and parent-of-origin effects in humans and animals. *Hum Mol Genet* 7(10), pp. 1599-1609.

Mostoslavsky, R., Alt, F. W. and Rajewsky, K. 2004. The lingering enigma of the allelic exclusion mechanism. *Cell* 118(5), pp. 539-544.

Murrell, A., Heeson, S. and Reik, W. 2004. Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. *Nat Genet* 36(8), pp. 889-893.

Nagahama, H., Hatakeyama, S., Nakayama, K., Nagata, M., Tomita, K. and Nakayama, K. 2001. Spatial and temporal expression patterns of the cyclin-dependent kinase (CDK) inhibitors p27Kip1 and p57Kip2 during mouse development. *Anat Embryol (Berl)* 203(2), pp. 77-87.

Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M. and Rossant, J. 1990. Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 110(3), pp. 815-821.

Nicholls, R. D., Knoll, J. H., Glatt, K., Hersh, J. H., Brewster, T. D., Graham, J. M., Jr., Wurster-Hill, D., Wharton, R. and Latt, S. A. 1989. Restriction fragment length polymorphisms within proximal 15q and their use in molecular cytogenetics and the Prader-Willi syndrome. *Am J Med Genet* 33(1), pp. 66-77.

Niswander, L. and Martin, G. R. 1992. Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* 114(3), pp. 755-768.

Nolan, C. M., Killian, J. K., Petitte, J. N. and Jirtle, R. L. 2001. Imprint status of M6P/IGF2R and IGF2 in chickens. *Dev Genes Evol* 211(4), pp. 179-183.

O'Neill, M. J. 2005. The influence of non-coding RNAs on allele-specific gene expression in mammals. *Hum Mol Genet* 14 Spec No 1, pp. R113-120.

Oakey, R. J. and Beechey, C. V. 2002. Imprinted genes: identification by chromosome rearrangements and post-genomic strategies. *Trends Genet* 18(7), pp. 359-366.

Okano, M., Bell, D. W., Haber, D. A. and Li, E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99(3), pp. 247-257.

Ong, K. K., Ahmed, M. L., Emmett, P. M., Preece, M. A. and Dunger, D. B. 2000. Association between postnatal catch-up growth and obesity in childhood: prospective cohort study. *Bmj* 320(7240), pp. 967-971.

Ono, R., Kobayashi, S., Wagatsuma, H., Aisaka, K., Kohda, T., Kaneko-Ishino, T. and Ishino, F. 2001. A retrotransposon-derived gene, PEG10, is a novel imprinted gene located on human chromosome 7q21. *Genomics* 73(2), pp. 232-237.

Ono, R., Nakamura, K., Inoue, K., Naruse, M., Usami, T., Wakisaka-Saito, N., Hino, T., Suzuki-Migishima, R., Ogonuki, N., Miki, H., Kohda, T., Ogura, A., Yokoyama, M., Kaneko-Ishino, T. and Ishino, F. 2006. Deletion of Peg10, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. *Nat Genet* 38(1), pp. 101-106.

Orr, H. A. 1995. Somatic mutation favors the evolution of diploidy. *Genetics* 139(3), pp. 1441-1447.

Osmond, C., Barker, D. J., Winter, P. D., Fall, C. H. and Simmonds, S. J. 1993. Early growth and death from cardiovascular disease in women. *Bmj* 307(6918), pp. 1519-1524.

Otto, S. P. and Goldstein, D. B. 1992. Recombination and the evolution of diploidy. *Genetics* 131(3), pp. 745-751.

Ozanne, S. E. and Hales, C. N. 2004. Lifespan: catch-up growth and obesity in male mice. *Nature* 427(6973), pp. 411-412.

Ozanne, S. E., Lewis, R., Jennings, B. J. and Hales, C. N. 2004. Early programming of weight gain in mice prevents the induction of obesity by a highly palatable diet. *Clin Sci (Lond)* 106(2), pp. 141-145.

Ozcelik, T., Leff, S., Robinson, W., Donlon, T., Lalande, M., Sanjines, E., Schinzel, A. and Francke, U. 1992. Small nuclear ribonucleoprotein polypeptide N (SNRPN), an expressed gene in the Prader-Willi syndrome critical region. *Nat Genet* 2(4), pp. 265-269.

Palmieri, S. L., Peter, W., Hess, H. and Scholer, H. R. 1994. Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev Biol* 166(1), pp. 259-267.

Parsons, T. J., Power, C. and Manor, O. 2001. Fetal and early life growth and body mass index from birth to early adulthood in 1958 British cohort: longitudinal study. *BMJ* 323(7325), pp. 1331-1335.

Pauler, F. M. and Barlow, D. P. 2006. Imprinting mechanisms--it only takes two. *Genes Dev* 20(10), pp. 1203-1206.

Pauler, F. M., Koerner, M. V. and Barlow, D. P. 2007. Silencing by imprinted noncoding RNAs: is transcription the answer? *Trends Genet* 23(6), pp. 284-292.

Paulsen, M., Davies, K. R., Bowden, L. M., Villar, A. J., Franck, O., Fuermann, M., Dean, W. L., Moore, T. F., Rodrigues, N., Davies, K. E., Hu, R. J., Feinberg, A. P., Maher, E. R., Reik, W. and Walter, J. 1998. Syntenic organization of the mouse distal chromosome 7 imprinting cluster and the Beckwith-Wiedemann syndrome region in chromosome 11p15.5. *Hum Mol Genet* 7(7), pp. 1149-1159.

Paulsen, M., El-Maarri, O., Engemann, S., Strodicke, M., Franck, O., Davies, K., Reinhardt, R., Reik, W. and Walter, J. 2000. Sequence conservation and variability of imprinting in the Beckwith-Wiedemann syndrome gene cluster in human and mouse. *Hum Mol Genet* 9(12), pp. 1829-1841.

Paulsen, M., Khare, T., Burgard, C., Tierling, S. and Walter, J. 2005. Evolution of the Beckwith-Wiedemann syndrome region in vertebrates. *Genome Res* 15(1), pp. 146-153.

Pedersen, R. A. 1986. *Potency, lineage and allocation in preimplantation mouse embryos* ***from*** *Experimental approaches to mammalian embryonic development.* Cambridge University Press.

Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T. and Collins, F. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 269(5223), pp. 540-543.

Peters, J. and Beechey, C. 2004. Identification and characterisation of imprinted genes in the mouse. *Brief Funct Genomic Proteomic* 2(4), pp. 320-333.

Petry, C. J., Dorling, M. W., Pawlak, D. B., Ozanne, S. E. and Hales, C. N. 2001. Diabetes in old male offspring of rat dams fed a reduced protein diet. *Int J Exp Diabetes Res* 2(2), pp. 139-143.

Pfeifer, K. 2000. Mechanisms of genomic imprinting. *Am J Hum Genet* 67(4), pp. 777-787.

Ping, A. J., Reeve, A. E., Law, D. J., Young, M. R., Boehnke, M. and Feinberg, A. P. 1989. Genetic linkage of Beckwith-Wiedemann syndrome to 11p15. *Am J Hum Genet* 44(5), pp. 720-723.

Plagge, A., Isles, A. R., Gordon, E., Humby, T., Dean, W., Gritsch, S., Fischer-Colbrie, R., Wilkinson, L. S. and Kelsey, G. 2005. Imprinted Nesp55 influences behavioral reactivity to novel environments. *Mol Cell Biol* 25(8), pp. 3019-3026.

Platz, E. and Newman, R. 2008. Diagnosis of IUGR: traditional biometry. *Semin Perinatol* 32(3), pp. 140-147.

Prawitt, D., Enklaar, T., Klemm, G., Gartner, B., Spangenberg, C., Winterpacht, A., Higgins, M., Pelletier, J. and Zabel, B. 2000. Identification and characterization of MTR1, a novel gene with homology to melastatin (MLSN1) and the trp gene family located in the BWS-WT2 critical region on chromosome 11p15.5 and showing allele-specific expression. *Hum Mol Genet* 9(2), pp. 203-216.

Qian, N., Frank, D., O'Keefe, D., Dao, D., Zhao, L., Yuan, L., Wang, Q., Keating, M., Walsh, C. and Tycko, B. 1997. The IPL gene on chromosome 11p15.5 is imprinted in humans and mice and is similar to TDAG51, implicated in Fas expression and apoptosis. *Hum Mol Genet* 6(12), pp. 2021-2029.

Rainier, S., Johnson, L. A., Dobry, C. J., Ping, A. J., Grundy, P. E. and Feinberg, A. P. 1993. Relaxation of imprinted genes in human cancer. *Nature* 362(6422), pp. 747-749.

Rampon, C., Prandini, M. H., Bouillot, S., Pointu, H., Tillet, E., Frank, R., Vernet, M. and Huber, P. 2005. Protocadherin 12 (VE-cadherin 2) is expressed in endothelial, trophoblast, and mesangial cells. *Exp Cell Res* 302(1), pp. 48-60.

Ravelli, A. C., van Der Meulen, J. H., Osmond, C., Barker, D. J. and Bleker, O. P. 1999. Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr* 70(5), pp. 811-816.

Rawn, S. M. and Cross, J. C. 2008. The evolution, regulation, and function of placenta-specific genes. *Annu Rev Cell Dev Biol* 24, pp. 159-181.

Redline, R. W., Chernicky, C. L., Tan, H. Q., Ilan, J. and Ilan, J. 1993. Differential expression of insulin-like growth factor-II in specific regions of the late (post day 9.5) murine placenta. *Mol Reprod Dev* 36(2), pp. 121-129.

Reik, W., Collick, A., Norris, M. L., Barton, S. C. and Surani, M. A. 1987. Genomic imprinting determines methylation of parental alleles in transgenic mice. *Nature* 328(6127), pp. 248-251.

Reik, W., Constância, M., Fowden, A., Anderson, N., Dean, W., Ferguson-Smith, A., Tycko, B. and Sibley, C. 2003. Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. *J Physiol* 547(Pt 1), pp. 35-44.

Reik, W., Dean, W. and Walter, J. 2001. Epigenetic reprogramming in mammalian development. *Science* 293(5532), pp. 1089-1093.

Reik, W. and Walter, J. 2001a. Evolution of imprinting mechanisms: the battle of the sexes begins in the zygote. *Nat Genet* 27(3), pp. 255-256.

Reik, W. and Walter, J. 2001b. Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2(1), pp. 21-32.

Renfree, M. B., Ager, E. I., Shaw, G. and Pask, A. J. 2008. Genomic imprinting in marsupial placentation. *Reproduction* 136(5), pp. 523-531.

Richardus, J. H., Graafmans, W. C., Verloove-Vanhorick, S. P. and Mackenbach, J. P. 2003. Differences in perinatal mortality and suboptimal care between 10 European regions: results of an international audit. *Bjog* 110(2), pp. 97-105.

Riley, P., Anson-Cartwright, L. and Cross, J. C. 1998. The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nat Genet* 18(3), pp. 271-275.

Ripoche, M. A., Kress, C., Poirier, F. and Dandolo, L. 1997. Deletion of the H19 transcription unit reveals the existence of a putative imprinting control element. *Genes Dev* 11(12), pp. 1596-1604.

Robertson, G., Garrick, D., Wu, W., Kearns, M., Martin, D. and Whitelaw, E. 1995. Position-dependent variegation of globin transgene expression in mice. *Proc Natl Acad Sci U S A* 92(12), pp. 5371-5375.

Rodriguez, T. A., Sparrow, D. B., Scott, A. N., Withington, S. L., Preis, J. I., Michalicek, J., Clements, M., Tsang, T. E., Shioda, T., Beddington, R. S. and Dunwoodie, S. L. 2004. Cited1 is required in trophoblasts for placental development and for embryo growth and survival. *Mol Cell Biol* 24(1), pp. 228-244.

Rossant, J. and Cross, J. C. 2001. Placental development: lessons from mouse mutants. *Nat Rev Genet* 2(7), pp. 538-548.

Rossant, J., Guillemot, F., Tanaka, M., Latham, K., Gertenstein, M. and Nagy, A. 1998. Mash2 is expressed in oogenesis and preimplantation development but is not required for blastocyst formation. *Mech Dev* 73(2), pp. 183-191.

Rougier, N. and Werb, Z. 2001. Minireview: Parthenogenesis in mammals. *Mol Reprod Dev* 59(4), pp. 468-474.

Saba-El-Leil, M. K., Vella, F. D., Vernay, B., Voisin, L., Chen, L., Labrecque, N., Ang, S. L. and Meloche, S. 2003. An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development. *EMBO Rep* 4(10), pp. 964-968.

Salas, M., John, R., Saxena, A., Barton, S., Frank, D., Fitzpatrick, G., Higgins, M. J. and Tycko, B. 2004. Placental growth retardation due to loss of imprinting of Phlda2. *Mech Dev* 121(10), pp. 1199-1210.

Sapienza, C., Peterson, A. C., Rossant, J. and Balling, R. 1987. Degree of methylation of transgenes is dependent on gamete of origin. *Nature* 328(6127), pp. 251-254.

Saxena, A., Morozov, P., Frank, D., Musalo, R., Lemmon, M. A., Skolnik, E. Y. and Tycko, B. 2002. Phosphoinositide binding by the pleckstrin homology domains of Ipl and Tih1. *J Biol Chem* 277(51), pp. 49935-49944.

Schoenherr, C. J., Levorse, J. M. and Tilghman, S. M. 2003. CTCF maintains differential methylation at the Igf2/H19 locus. *Nat Genet* 33(1), pp. 66-69.

Schulz, R., Menheniott, T. R., Woodfine, K., Wood, A. J., Choi, J. D. and Oakey, R. J. 2006. Chromosome-wide identification of novel imprinted genes using microarrays and uniparental disomies. *Nucleic Acids Res* 34(12), p. e88.

Scott, I. C., Anson-Cartwright, L., Riley, P., Reda, D. and Cross, J. C. 2000. The HAND1 basic helix-loop-helix transcription factor regulates trophoblast differentiation via multiple mechanisms. *Mol Cell Biol* 20(2), pp. 530-541.

Screen, M., Dean, W., Cross, J. C. and Hemberger, M. 2008. Cathepsin proteases have distinct roles in trophoblast function and vascular remodelling. *Development* 135(19), pp. 3311-3320.

Searle, A. G. and Beechey, C. V. 1990. Genome imprinting phenomena on mouse chromosome 7. *Genet Res* 56(2-3), pp. 237-244.

Sekita, Y., Wagatsuma, H., Nakamura, K., Ono, R., Kagami, M., Wakisaka, N., Hino, T., Suzuki-Migishima, R., Kohda, T., Ogura, A., Ogata, T., Yokoyama, M., Kaneko-Ishino, T. and Ishino, F. 2008. Role of retrotransposon-derived imprinted gene, Rtl1, in the feto-maternal interface of mouse placenta. *Nat Genet* 40(2), pp. 243-248.

Sibilia, M. and Wagner, E. F. 1995. Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* 269(5221), pp. 234-238.

Sibley, C. P., Coan, P. M., Ferguson-Smith, A. C., Dean, W., Hughes, J., Smith, P., Reik, W., Burton, G. J., Fowden, A. L. and Constância, M. 2004. Placental-specific insulin-like growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta. *Proc Natl Acad Sci U S A* 101(21), pp. 8204-8208.

Sifianou, P. 2006. Small and growth-restricted babies: drawing the distinction. *Acta Paediatr* 95(12), pp. 1620-1624.

Simmons, D. G. and Cross, J. C. 2005. Determinants of trophoblast lineage and cell subtype specification in the mouse placenta. *Dev Biol* 284(1), pp. 12-24.

Simmons, D. G., Fortier, A. L. and Cross, J. C. 2007. Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. *Dev Biol* 304(2), pp. 567-578.

Simmons, D. G., Natale, D. R., Begay, V., Hughes, M., Leutz, A. and Cross, J. C. 2008a. Early patterning of the chorion leads to the trilaminar trophoblast cell structure in the placental labyrinth. *Development* 135(12), pp. 2083-2091.

Simmons, D. G., Rawn, S., Davies, A., Hughes, M. and Cross, J. C. 2008b. Spatial and temporal expression of the 23 murine Prolactin/Placental Lactogen-related genes is not associated with their position in the locus. *BMC Genomics* 9, p. 352.

Simmons, R. A., Templeton, L. J. and Gertz, S. J. 2001. Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. *Diabetes* 50(10), pp. 2279-2286.

Smilinich, N. J., Day, C. D., Fitzpatrick, G. V., Caldwell, G. M., Lossie, A. C., Cooper, P. R., Smallwood, A. C., Joyce, J. A., Schofield, P. N., Reik, W., Nicholls, R. D., Weksberg, R., Driscoll, D. J., Maher, E. R., Shows, T. B. *et al.* 1999. A maternally methylated CpG island in KvLQT1 is associated with an

antisense paternal transcript and loss of imprinting in Beckwith-Wiedemann syndrome. *Proc Natl Acad Sci U S A* 96(14), pp. 8064-8069.

Smith, R. J., Dean, W., Konfortova, G. and Kelsey, G. 2003. Identification of novel imprinted genes in a genome-wide screen for maternal methylation. *Genome Res* 13(4), pp. 558-569.

Solter, D. 1994. Refusing the ovarian time bomb. *Trends Genet* 10(10), pp. 346; author reply 348-349.

Sorhede Winzell, M. and Ahren, B. 2004. Glucagon-like peptide-1 and islet lipolysis. *Horm Metab Res* 36(11-12), pp. 795-803.

Spahn, L. and Barlow, D. P. 2003. An ICE pattern crystallizes. *Nat Genet* 35(1), pp. 11-12.

Strumpf, D., Mao, C. A., Yamanaka, Y., Ralston, A., Chawengsaksophak, K., Beck, F. and Rossant, J. 2005. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* 132(9), pp. 2093-2102.

Sturm, K. S., Flannery, M. L. and Pedersen, R. A. 1994. Abnormal development of embryonic and extraembryonic cell lineages in parthenogenetic mouse embryos. *Dev Dyn* 201(1), pp. 11-28.

Sun, F. L., Dean, W. L., Kelsey, G., Allen, N. D. and Reik, W. 1997. Transactivation of Igf2 in a mouse model of Beckwith-Wiedemann syndrome. *Nature* 389(6653), pp. 809-815.

Surani, M. A. 1998. Imprinting and the initiation of gene silencing in the germ line. *Cell* 93(3), pp. 309-312.

Surani, M. A., Barton, S. C. and Norris, M. L. 1984. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* 308(5959), pp. 548-550.

Surani, M. A., Barton, S. C. and Norris, M. L. 1986. Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome. *Cell* 45(1), pp. 127-136.

Surani, M. A., Barton, S. C. and Norris, M. L. 1987. Influence of parental chromosomes on spatial specificity in androgenetic----parthenogenetic chimaeras in the mouse. *Nature* 326(6111), pp. 395-397.

Surwit, R. S., Kuhn, C. M., Cochrane, C., McCubbin, J. A. and Feinglos, M. N. 1988. Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* 37(9), pp. 1163-1167.

Suzuki, S., Renfree, M. B., Pask, A. J., Shaw, G., Kobayashi, S., Kohda, T., Kaneko-Ishino, T. and Ishino, F. 2005. Genomic imprinting of IGF2, p57(KIP2)

and PEG1/MEST in a marsupial, the tammar wallaby. *Mech Dev* 122(2), pp. 213-222.

Swain, J. L., Stewart, T. A. and Leder, P. 1987. Parental legacy determines methylation and expression of an autosomal transgene: a molecular mechanism for parental imprinting. *Cell* 50(5), pp. 719-727.

Szabo, P., Tang, S. H., Rentsendorj, A., Pfeifer, G. P. and Mann, J. R. 2000. Maternal-specific footprints at putative CTCF sites in the H19 imprinting control region give evidence for insulator function. *Curr Biol* 10(10), pp. 607-610.

Szabo, P. E. and Mann, J. R. 1995. Biallelic expression of imprinted genes in the mouse germ line: implications for erasure, establishment, and mechanisms of genomic imprinting. *Genes Dev* 9(15), pp. 1857-1868.

Takagi, N. and Sasaki, M. 1975. Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. *Nature* 256(5519), pp. 640-642.

Takahashi, K., Kobayashi, T. and Kanayama, N. 2000a. p57(Kip2) regulates the proper development of labyrinthine and spongiotrophoblasts. *Mol Hum Reprod* 6(11), pp. 1019-1025.

Takahashi, K., Nakayama, K. and Nakayama, K. 2000b. Mice lacking a CDK inhibitor, p57Kip2, exhibit skeletal abnormalities and growth retardation. *J Biochem (Tokyo)* 127(1), pp. 73-83.

Tanaka, M., Gertsenstein, M., Rossant, J. and Nagy, A. 1997. Mash2 acts cell autonomously in mouse spongiotrophoblast development. *Dev Biol* 190(1), pp. 55-65.

Tanaka, S., Oda, M., Toyoshima, Y., Wakayama, T., Tanaka, M., Yoshida, N., Hattori, N., Ohgane, J., Yanagimachi, R. and Shiota, K. 2001. Placentomegaly in cloned mouse concepti caused by expansion of the spongiotrophoblast layer. *Biol Reprod* 65(6), pp. 1813-1821.

Tang, K. F., Wang, Y., Wang, P., Chen, M., Chen, Y., Hu, H. D., Hu, P., Wang, B., Yang, W. and Ren, H. 2007. Upregulation of PHLDA2 in Dicer knockdown HEK293 cells. *Biochim Biophys Acta*.

Tarkowski, A. K., Witkowska, A. and Nowicka, J. 1970. Experimental partheonogenesis in the mouse. *Nature* 226(5241), pp. 162-165.

Tartaglia, L. A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K. J., Smutko, J. S. *et al.* 1995. Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83(7), pp. 1263-1271.

Te Velde, S. J., Twisk, J. W., Van Mechelen, W. and Kemper, H. C. 2003. Birth weight, adult body composition, and subcutaneous fat distribution. *Obes Res* 11(2), pp. 202-208.

Thakur, N., Tiwari, V. K., Thomassin, H., Pandey, R. R., Kanduri, M., Gondor, A., Grange, T., Ohlsson, R. and Kanduri, C. 2004. An antisense RNA regulates the bidirectional silencing property of the Kcnq1 imprinting control region. *Mol Cell Biol* 24(18), pp. 7855-7862.

Thomson, J. A. and Solter, D. 1988. The developmental fate of androgenetic, parthenogenetic, and gynogenetic cells in chimeric gastrulating mouse embryos. *Genes Dev* 2(10), pp. 1344-1351.

Thorvaldsen, J. L., Duran, K. L. and Bartolomei, M. S. 1998. Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. *Genes Dev* 12(23), pp. 3693-3702.

Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R. C. and et al. 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* 269(5221), pp. 230-234.

Tremblay, K. D., Duran, K. L. and Bartolomei, M. S. 1997. A 5' 2-kilobase-pair region of the imprinted mouse H19 gene exhibits exclusive paternal methylation throughout development. *Mol Cell Biol* 17(8), pp. 4322-4329.

Tsang, P., Gilles, F., Yuan, L., Kuo, Y. H., Lupu, F., Samara, G., Moosikasuwan, J., Goye, A., Zelenetz, A. D., Selleri, L. and et al. 1995. A novel L23-related gene 40 kb downstream of the imprinted H19 gene is biallelically expressed in mid-fetal and adult human tissues. *Hum Mol Genet* 4(9), pp. 1499-1507.

Tucker, K. L., Beard, C., Dausmann, J., Jackson-Grusby, L., Laird, P. W., Lei, H., Li, E. and Jaenisch, R. 1996. Germ-line passage is required for establishment of methylation and expression patterns of imprinted but not of nonimprinted genes. *Genes Dev* 10(8), pp. 1008-1020.

Tycko, B. 2006. Imprinted genes in placental growth and obstetric disorders. *Cytogenet Genome Res* 113(1-4), pp. 271-278.

Tycko, B. and Morison, I. M. 2002. Physiological functions of imprinted genes. *J Cell Physiol* 192(3), pp. 245-258.

Tzortzaki, E. G., Yang, M., Glass, D., Deng, L., Evan, A. P., Bledsoe, S. B., Stambrook, P. J., Sahota, A. and Tischfield, J. A. 2003. Impaired expression of an organic cation transporter, IMPT1, in a knockout mouse model for kidney stone disease. *Urol Res* 31(4), pp. 257-261.

Valsamakis, G., Kanaka-Gantenbein, C., Malamitsi-Puchner, A. and Mastorakos, G. 2006. Causes of intrauterine growth restriction and the postnatal development of the metabolic syndrome. *Ann N Y Acad Sci* 1092, pp. 138-147.

Vandenbosche, R. C. and Kirchner, J. T. 1998. Intrauterine growth retardation. *Am Fam Physician* 58(6), pp. 1384-1390, 1393-1384.

Varmuza, S. and Mann, M. 1994. Genomic imprinting--defusing the ovarian time bomb. *Trends Genet* 10(4), pp. 118-123.

Verrey, F., Closs, E. I., Wagner, C. A., Palacin, M., Endou, H. and Kanai, Y. 2004. CATs and HATs: the SLC7 family of amino acid transporters. *Pflugers Arch* 447(5), pp. 532-542.

Verrey, F., Jack, D. L., Paulsen, I. T., Saier, M. H., Jr. and Pfeiffer, R. 1999. New glycoprotein-associated amino acid transporters. *J Membr Biol* 172(3), pp. 181-192.

Verrey, F., Meier, C., Rossier, G. and Kuhn, L. C. 2000. Glycoprotein-associated amino acid exchangers: broadening the range of transport specificity. *Pflugers Arch* 440(4), pp. 503-512.

Vickers, M. H., Breier, B. H., Cutfield, W. S., Hofman, P. L. and Gluckman, P. D. 2000. Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *Am J Physiol Endocrinol Metab* 279(1), pp. E83-87.

Villar, J., Smeriglio, V., Martorell, R., Brown, C. H. and Klein, R. E. 1984. Heterogeneous growth and mental development of intrauterine growth-retarded infants during the first 3 years of life. *Pediatrics* 74(5), pp. 783-791.

Vinkenoog, R., Bushell, C., Spielman, M., Adams, S., Dickinson, H. G. and Scott, R. J. 2003. Genomic imprinting and endosperm development in flowering plants. *Mol Biotechnol* 25(2), pp. 149-184.

Wakisaka, N., Inoue, K., Ogonuki, N., Miki, H., Sekita, Y., Hanaki, K., Akatsuka, A., Kaneko-Ishino, T., Ishino, F. and Ogura, A. 2008. Ultrastructure of placental hyperplasia in mice: comparison of placental phenotypes with three different etiologies. *Placenta* 29(8), pp. 753-759.

Walsh, C., Glaser, A., Fundele, R., Ferguson-Smith, A., Barton, S., Surani, M. A. and Ohlsson, R. 1994. The non-viability of uniparental mouse conceptuses correlates with the loss of the products of imprinted genes. *Mech Dev* 46(1), pp. 55-62.

Wang, X., Sun, Q., McGrath, S. D., Mardis, E. R., Soloway, P. D. and Clark, A. G. 2008. Transcriptome-wide identification of novel imprinted genes in neonatal mouse brain. *PLoS ONE* 3(12), p. e3839.

Watanabe, D. and Barlow, D. P. 1996. Random and imprinted monoallelic expression. *Genes Cells* 1(9), pp. 795-802.

Watson, E. D. and Cross, J. C. 2005. Development of structures and transport functions in the mouse placenta. *Physiology (Bethesda)* 20, pp. 180-193.

Watts, P. C., Buley, K. R., Sanderson, S., Boardman, W., Ciofi, C. and Gibson, R. 2006. Parthenogenesis in Komodo dragons. *Nature* 444(7122), pp. 1021-1022.

Weksberg, R., Shen, D. R., Fei, Y. L., Song, Q. L. and Squire, J. 1993. Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome. *Nat Genet* 5(2), pp. 143-150.

West, J. D., Frels, W. I., Chapman, V. M. and Papaioannou, V. E. 1977. Preferential expression of the maternally derived X chromosome in the mouse yolk sac. *Cell* 12(4), pp. 873-882.

Westbury, J., Watkins, M., Ferguson-Smith, A. C. and Smith, J. 2001. Dynamic temporal and spatial regulation of the cdk inhibitor p57(kip2) during embryo morphogenesis. *Mech Dev* 109(1), pp. 83-89.

Westerman, B. A., Poutsma, A., Looijenga, L. H., Wouters, D., van Wijk, I. J. and Oudejans, C. B. 2001. The Human Achaete Scute Homolog 2 gene contains two promotors, generating overlapping transcripts and encoding two proteins with different nuclear localization. *Placenta* 22(6), pp. 511-518.

Wu, Q., Ohsako, S., Ishimura, R., Suzuki, J. S. and Tohyama, C. 2004. Exposure of mouse preimplantation embryos to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters the methylation status of imprinted genes H19 and Igf2. *Biol Reprod* 70(6), pp. 1790-1797.

Wynne, F., Ball, M., McLellan, A. S., Dockery, P., Zimmermann, W. and Moore, T. 2006. Mouse pregnancy-specific glycoproteins: tissue-specific expression and evidence of association with maternal vasculature. *Reproduction* 131(4), pp. 721-732.

Xie, T., Plagge, A., Gavrilova, O., Pack, S., Jou, W., Lai, E. W., Frontera, M., Kelsey, G. and Weinstein, L. S. 2006. The alternative stimulatory G protein alpha-subunit XLalphas is a critical regulator of energy and glucose metabolism and sympathetic nerve activity in adult mice. *J Biol Chem* 281(28), pp. 18989-18999.

Xin, Z., Soejima, H., Higashimoto, K., Yatsuki, H., Zhu, X., Satoh, Y., Masaki, Z., Kaneko, Y., Jinno, Y., Fukuzawa, R., Hata, J. and Mukai, T. 2000. A novel imprinted gene, KCNQ1DN, within the WT2 critical region of human chromosome 11p15.5 and its reduced expression in Wilms' tumors. *J Biochem* 128(5), pp. 847-853.

Yamaguchi, M., Ogren, L., Endo, H., Thordarson, G., Bigsby, R. M. and Talamantes, F. 1992. Production of mouse placental lactogen-I and placental lactogen-II by the same giant cell. *Endocrinology* 131(4), pp. 1595-1602.

Yan, Y., Frisen, J., Lee, M. H., Massague, J. and Barbacid, M. 1997. Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev* 11(8), pp. 973-983.

Yoder, J. A., Walsh, C. P. and Bestor, T. H. 1997. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet* 13(8), pp. 335-340.

Yoshihashi, H., Maeyama, K., Kosaki, R., Ogata, T., Tsukahara, M., Goto, Y., Hata, J., Matsuo, N., Smith, R. J. and Kosaki, K. 2000. Imprinting of human GRB10 and its mutations in two patients with Russell-Silver syndrome. *Am J Hum Genet* 67(2), pp. 476-482.

Yu, S., Gavrilova, O., Chen, H., Lee, R., Liu, J., Pacak, K., Parlow, A. F., Quon, M. J., Reitman, M. L. and Weinstein, L. S. 2000. Paternal versus maternal transmission of a stimulatory G-protein alpha subunit knockout produces opposite effects on energy metabolism. *J Clin Invest* 105(5), pp. 615-623.

Zechner, U., Hemberger, M., Constancia, M., Orth, A., Dragatsis, I., Luttges, A., Hameister, H. and Fundele, R. 2002. Proliferation and growth factor expression in abnormally enlarged placentas of mouse interspecific hybrids. *Dev Dyn* 224(2), pp. 125-134.

Zechner, U., Reule, M., Orth, A., Bonhomme, F., Strack, B., Guenet, Hameister, H. and Fundele, R. 1996. An X-chromosome linked locus contributes to abnormal placental development in mouse interspecific hybrid. *Nat Genet* 12(4), pp. 398-403.

Zhang, P., Liegeois, N. J., Wong, C., Finegold, M., Hou, H., Thompson, J. C., Silverman, A., Harper, J. W., DePinho, R. A. and Elledge, S. J. 1997. Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. *Nature* 387(6629), pp. 151-158.

Ziomek, C. A., Johnson, M. H. and Handyside, A. H. 1982. The developmental potential of mouse 16-cell blastomeres. *J Exp Zool* 221(3), pp. 345-355.

Zubair, M., Hilton, K., Saam, J. R., Surani, M. A., Tilghman, S. M. and Sasaki, H. 1997. Structure and expression of the mouse L23mrp gene downstream of the imprinted H19 gene: biallelic expression and lack of interaction with the H19 enhancers. *Genomics* 45(2), pp. 290-296.

Zwart, R., Sleutels, F., Wutz, A., Schinkel, A. H. and Barlow, D. P. 2001. Bidirectional action of the Igf2r imprint control element on upstream and downstream imprinted genes. *Genes Dev* 15(18), pp. 2361-2366.