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RNA Interference in ES cells

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at

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by

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Summary

The aim of this project was to develop methods for conditional induction of gene specific silencing in murine embryonic stem (mES) cells using RNA interference (RNAi). RNA interference (RNAi) is a process whereby double stranded (ds) RNA can reduce the expression of a gene by selective mRNA degradation. This thesis describes the investigation of several methods of inducing RNAi in mES cells. These included design and transfection of chemically synthesised small interfering (si) RNAs; the transfection of vectors expressing short hairpin (sh) RNAs and construction of a vector that expresses hairpin dsRNA under tetracycline control. A specific reduction in GFP expression was observed by the transfection of siRNAs targeting either plasmid or endogenously expressed GFP. Transfections of two out of three Oct-4 siRNAs were shown to reduce the expression of *Oct-4*. mES cell lines were made that stably express shRNAs targeting *Rex-1* and *Laminin B1*. Analysis of gene expression by semi-quantitative RT-PCR showed knockdown of the target genes in some cell lines. Transfection of another vector expressing Oct-4 shRNA was shown to induce differentiation of mES cells as measured by an increase in cell size. Methods were developed for the cloning of an inverted-repeat of target genes (*Oct-4*, *LMNA*) into a tetracycline-inducible vector. The system was tested in a HEK 293 cell line expressing the tet-responsive transactivator protein. The hairpin dsRNA was shown to be expressed when doxycycline was added to the cells but no knockdown of *LMNA* was observed. The use of siRNAs and shRNAs to induce gene specific silencing in ES cells was shown. Although fast and efficient methods for the assaying of RNAi induced knockdown in cells were not demonstrated. Therefore the potential of RNAi as a high throughput system for establishing gene function in mES has yet to be realised.

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Abbreviations and Conventions

The following were used throughout the text.

A	Adenosine
A _(x)	Absorbance at x nm
ADP	Adenosine Di-Phosphate
ATP	Adenosine Tri-Phosphate
Blast	Basic local Alignment search Tool
bp	Nucleotide base pairs
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
CMV	Cytomegalovirus
dATP	Dexoyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
ds	Double-stranded
dsRBM	DsRNA binding motif
DTT	Dithiothreitol
EC	Embryonal carcinoma cells
EDTA	Ethylene diamine tetracetic acid
EGFP	Enhanced green fluorescent protein
ES	Embryonic stem cells
EST	Expressed sequence tag
FCS	Foetal calf serum
g	Gravity
G	Guanine
gDNA	Genomic DNA
GFP	Green fluorescent protein
h	Hour

Abbreviations and Conventions

HEK	Human embryonal kidney cells
HEPES	(N-[2-HydroxyEthyl]-Piperazine-N'-[2-EthaneSulphonic acid])
hES	Human ES cells
hp	Hairpin
IR	Inverted repeat
kb	Nucleotide kilobase pairs
LB	Luria Broth (Luria Bertani media)
LIF	Leukaemia Inhibitory Factor
M	Molar concentration
m	milli 10 ⁻³
MCS	Multiple cloning site
mES	Mouse ES cells
miRNA	Micro RNA
min	Minute
MmGFP	Modified form of GFP
MMLV	Moloney-Murine Leukaemia Virus
mRNA	Messenger RNA
NBS	Newborn calf serum
nm	Nanometres
nt	Nucleotide
OD	Optical density
oligo	Oligonucleotide
o/n	Over night
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
RT-PCR	Reverse-transcription-polymerase chain reaction
SDS	Sodium dodecyl sulphate
secs	Seconds
shRNA	Short hairpin RNA
siRNA	Small interfering RNA

Abbreviations and Conventions

ss	Single stranded
T	Thymine
TAE	Tris Acetate-EDTA buffer
TE	Tris-EDTA buffer
Tet	Tetracycline
Tris	N-Tris[HydroxyMethyl]methylglycine;N-[2-Hydroxy-1,1-bis(hydroxymethy)-ethyl]glycine
U	Uracil
UTR	Untranslated region
UV	Ultraviolet
w/v	Weight by volume

All laboratory materials employed are detailed according to their commercial name given in the appropriate manufacturer's catalogue.

Glossary of RNAi terms

Dicer	RNase III nuclease, cleaves dsRNAs and pre-miRNAs to siRNAs and miRNAs respectively.
dsRNA	Long double-stranded RNA molecules.
hpRNA	Longer dsRNA expressed from a vector that contains an inverted repeat of part of the cDNA sequence of a gene of interest separated by a spacer sequence. This is transcribed to form a hairpin RNA.
Interferon Response	Response to dsRNA resulting in non-specific mRNA degradation and inhibition of protein synthesis.
miRNA	MicroRNAs are naturally occurring small non-coding RNA sequences, (~21 nucleotides) many involved in regulating development.
Off target effects	A decrease in gene expression of genes other than the target gene induced by dsRNA or siRNA transfection.
Pri-miRNA	Long primary transcripts that are subsequently cleaved by an enzyme called Drosha to shorter 70 nucleotide stem loop precursors called pre-miRNAs.
Pre-miRNA	See above. These are processed by Dicer to mature miRNAs.
RISC	RNA induced silencing complex. A single strand of siRNA or miRNA is incorporated into RISC. The RISC:miRNA/siRNA complex then binds to its target sequence resulting in mRNA cleavage or translational repression.

- RNAi** RNA interference is a process whereby dsRNA can reduce the expression of a gene by selective mRNA degradation [term coined by Fire et al. (Fire, Xu et al. 1998)].
- shRNA** Short hairpin RNA contains a sense and antisense strand of the target mRNA sequences (~21 nucleotides) separated by a short spacer region. The shRNA is usually expressed in mammalian cells from a vector containing a polymerase III promoter.
- siRNA** Short interfering RNAs are short (~21 nucleotide) duplexes with 2 nucleotide 3' overhanging ends. They are produced by Dicer or can be chemically synthesised.

Summary	III
Acknowledgements	IV
Abbreviations and Conventions	V
Glossary of RNAi Terms	VIII
1. Introduction	1
1.1. RNAi Interference	3
1.1.1. Mechanism of RNAi	3
1.1.1.a. miRNAs	7
1.1.1.b. Dicer	10
1.1.1.c. RISC	11
1.1.2. Previous Studies	16
1.1.2.a. RNAi in non-mammalian cells	17
1.1.2.b. RNAi in mammalian cells	18
1.1.3. Present and potential applications of RNAi	21
1.1.3.a. RNAi Libraries	22
1.1.3.b. Generation of mouse models	24
1.1.3.c. Treatment of diseases and viral infections	24
1.1.3.d. Cancer Therapy	26
1.2. Murine Embryonic Stem cells	26
1.2.1. mES cell self-renewal	27
1.2.2. Genes known to be involved in self-renewal of mES cells	30
1.2.2.a. Oct-4	30
1.2.2.b. Nanog	30
1.2.2.c. Sox-2	31
1.3. Aims of the Project	32
2. Materials and Methods	33
2.1. Materials	34
2.1.1. Autoclaving Conditions	35
2.1.2. Medium preparation	35
2.1.2.a. For maintaining mammalian cells	35
2.1.2.b. For bacterial culture	36
2.1.3. Antibiotics	37
2.1.4. DNA Markers	37
2.1.5. Oligonucleotide primers	37
2.1.6. Reagents and Buffers	39
2.2. Tissue Culture	42
2.2.1. Maintenance of cells	42
2.2.1.a. Thawing cells	42
2.2.1.b. Passaging of cells	42
2.2.1.c. Freezing down cells for storage in liquid nitrogen	43

2.2.2. Transfection methods	43
2.2.2.a. Lipofectamine™ 2000 transfection	43
2.2.2.b. Calcium phosphate transfection	44
2.2.2.c. Electroporation of cells	44
2.2.3. Determining the optimum concentration of selection reagent	45
2.2.4. Picking ES cell colonies of stably transformed cells	45
2.2.5. Culturing resistant cell clones	46
2.3. Molecular Biology	46
2.3.1. Agarose Gels	46
2.3.2. Preparation of cytoplasmic RNA	47
2.3.3. DNase treatment of RNA	47
2.3.4. Reverse Transcription	48
2.3.5. PCR Amplification	48
2.3.6. Plasmid DNA preparation	49
2.3.6.a. Small scale preparation of plasmid DNA	49
2.3.6.b. Large scale preparation of plasmid DNA	49
2.3.7. Genomic DNA preparation from cells	50
2.3.8. Quantification of nucleic acid concentrations	51
2.3.9. Annealing of oligonucleotides	51
2.3.10. Restriction Digests	51
2.3.10.a. Analytical	51
2.3.10.b. Preparative Restriction Digest	52
2.3.11. Extraction of DNA from agarose gels	52
2.3.12. Purification of DNA	53
2.3.13. Ligation of insert DNA into digested plasmid	53
2.3.14. Preparation of chemically competent <i>E. coli</i> cells	54
2.3.15. Transformation of chemically competent <i>E. coli</i> bacteria	54
2.3.16. PCR Screening of bacterial colonies	54
2.3.17. DNA Sequencing Using the ABI 3100 System	55
3. RNAi induced gene silencing using siRNAs	56
3.1. Introduction	57
3.2. Experimental approach and results	58
3.2.1. Sequencing of MmGFP	58
3.2.2. siRNA Design	59
3.2.3. Annealing of siRNA oligos	61
3.2.4. Co-transfections of MmGFP siRNAs and MmGFP expressing plasmid	61
3.2.5. Knockdown of endogenously expressed MmGFP in mES cells	63
3.2.6. Knockdown of Oct-4 in mES cells	65
3.3. Discussion	68
4. RNAi induced gene silencing using vectors expressing shRNAs	74
4.1. Introduction	75
4.2. Experimental Approach and Results	76
4.2.1. psiRNA-hH1zeo	76
4.2.2. psi vector	82
4.3. Discussion	90

<u>5. Inducible expression of hairpin dsRNA for gene specific silencing</u>	92
5.1. Introduction	93
5.2. Experimental Approach and Results	96
5.2.1. Construction of pBI-EGFP vector that expresses hairpin dsRNA	96
5.2.1.a. Modifications to the pBI-EGFP Vector	96
5.2.1.b. Cloning of the Oct-4 cDNA as an inverted repeat into pBI-EGFP*	101
5.2.2. Construction of an ES cell line expressing rtTA	108
5.2.3. Construction and transfection of the pBI-EGFP expressing hpRNA targeting LaminA/C in 293 Tet-On cells	110
5.3. Discussion	115
<u>6. General Discussion</u>	119
6.1. Use of siRNAs for elucidating gene function in mES cells	120
6.2. Transfection of plasmids expressing shRNA into mES cells	121
6.3. Inducible systems for gene specific silencing by RNAi	123
6.4. Current literature on the use of RNAi for studying gene function in mES cells and mouse models	125
6.5. Conclusion	133
<u>Bibliography</u>	135
<u>Appendix 1: Vector Maps</u>	157
A1.1. pBI-EGFP	158
A1.2. psiRNA-hH1zeo	159
A1.3. psiOct-4	160
A1.4. pTet-On	161
A1.5. pTP6	162
<u>Appendix 2: Sequences of regions targeted for RNAi</u>	163
A2.1 Oct-4	164
A2.2. Rex-1	165
A2.3 Laminin B1 (<i>Lamb1</i>)	166
A2.4 Lamin A/C (<i>LMNA</i>)	167
<u>Appendix 3: Cell Area Measurements</u>	168

Chapter 1

General Introduction

1. Introduction

The first draft sequences of the human genome were published in 2001 (Lander *et al.*, 2001; Venter *et al.*, 2001). The conversion of draft data into a highly accurate sequence covering ~99% of the euchromatic genome was published 3 years later (2004). This information has been utilised for the identification of potential gene coding regions, [the number predicted to be between 20,000-25,000 (2004)], studies of the chromosomal structure and organisation and also the distribution of polymorphisms. Comparisons between human and mouse genomes have allowed evolutionarily conserved genes to be identified (Waterston *et al.*, 2002). Although bioinformatics have been used to identify the position and number of genes, the function of many of these genes has yet to be determined.

Determining the function of the large number of uncharacterised genes remains the challenge of our era. Progress has been made via the use of high throughput techniques, such as microarrays and proteomic technologies. To date, however the 'gold standard' for determination of gene function remains the generation of knockout and transgenic mice [for review see (Houdebine, 2002)]. Despite the success of mouse models for determining gene function *in vivo*, their production remains expensive, time consuming and labour intensive. Antisense nucleic acid derivatives including ribozymes and antisense oligonucleotides have been investigated as potentially faster, simpler and cheaper alternatives for determining gene function (Lebedeva and Stein, 2001).

Introduction of an antisense oligonucleotide downregulates the expression of the gene whose mRNA is complementary to the oligonucleotide sequence. Fire and colleagues investigated the requirements of the RNA delivered in *Caenorhabditis elegans* and discovered that double stranded RNA (dsRNA) was 10 fold more effective at silencing than either sense or antisense single stranded RNAs (Fire *et al.*, 1998). This process was named RNA interference (RNAi). This was similar to a gene silencing effect that been previously observed in plants, named cosuppression (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Overexpression of chalcone synthase, an enzyme involved in the synthesis of the pigment of petunias, was predicted to enhance floral colouration. The results were very different, the flowers were actually found to be

totally white or variegated. This gene silencing phenomenon was also observed in fungi and termed quelling (Romano and Macino, 1992). During attempts to increase the amount of orange pigment in *Neurospora crassa* a wildtype strain was transformed with extra copies of a gene involved in making carotenoid pigment (albino-1 and albino-3). The presence of these extra copies was shown to suppress the expression of the endogenous al-1 or al-3 genes with up to 36% of transformants showing an albino phenotype.

1.1. RNAi Interference

RNAi is a process whereby double-stranded (ds) RNA reduces (or 'knocks-down') the expression of a gene by selective mRNA degradation. RNAi has been shown to occur in many organisms including *C. elegans* (Fire *et al.*, 1998; Ketting and Plasterk, 2000), *Drosophila* (Kennerdell and Carthew, 2000), and mice (McCaffrey *et al.*, 2002; Svoboda *et al.*, 2000; Svoboda *et al.*, 2001; Wianny and Zernicka-Goetz, 2000) and also in plants (Voinnet *et al.*, 1998; Waterhouse *et al.*, 1998). RNAi is thought to protect an organism from viral infections and mobile genetic elements (Plasterk, 2002).

The potential of RNAi for large scale functional genomics has been shown with the development of RNAi libraries that were used for screening and discovering the function of many genes in *C. elegans* (Fraser *et al.*, 2000). Also more recently several studies have shown its potential use in treatment of heart disease (Soutschek *et al.*, 2004), viruses (Capodici *et al.*, 2002) and also cancer therapy (Yague *et al.*, 2004). These will be discussed later in this chapter.

1.1.1. Mechanism of RNAi

Double-stranded RNA (dsRNA) introduced into cells is first processed to shorter RNA duplexes of about 22 nucleotides (nt) by an RNase III nuclease named Dicer (Bernstein *et al.*, 2001) (Figure 1.1). These short RNA duplexes were named small interfering (si) RNAs (Elbashir *et al.*, 2001b). The RNase III nuclease activity of Dicer cleaves both stands of the dsRNA to give siRNA duplexes with 2 nt 3' overhanging ends (Elbashir *et al.*, 2001b). It was suggested that the use of thymidine

overhang at the 3' end helps prevent degradation of the siRNA by exonucleases (Elbashir *et al.*, 2001c).

The siRNAs then act in conjunction with a multimeric protein complex named the RNA-induced silencing complex (RISC) (Hammond *et al.*, 2000). Interaction with RISC results in cleavage of target RNA in the centre of the region that is homologous to the siRNA between residues 10 and 11 as counted from the 5' end (Elbashir *et al.*, 2001b). This cleavage does not require ATP (Nykanen *et al.*, 2001). Cleaved target RNA is degraded quickly due to loss of either the stabilising cap or poly(A) tail (Elbashir *et al.*, 2001c).

Mammalian cells also exhibit an interferon response (Stark *et al.*, 1998) when dsRNA of greater than 30 base pairs (bp) are introduced (Figure 1.1). Two enzymes are activated that are normally induced by interferon. The first is the dsRNA-dependent protein kinase (PKR) that phosphorylates translation initiation factor eIF2a, inhibiting protein synthesis. PKR is not activated by dsRNA of less than 30 bp (Manche *et al.*, 1992). The second enzyme activated is 2' 5' oligoadenylate synthetase that synthesises 2' 5' oligoadenylate. 2' 5' oligoadenylate binds to and activates RNaseL, resulting in non-specific mRNA degradation (Stark *et al.*, 1998). This interferon response is present to protect the host genome from invading viruses. It was thought that specific RNAi can be achieved in mammalian cells containing the interferon response by the use of siRNAs (Elbashir *et al.*, 2001a). These shorter 21-22 nt duplex RNA sequences should not activate the interferon response.

Subsequent studies have shown that the interferon response can be still triggered when using siRNAs. Sledz and colleagues studied the non-specific effects of transfection of siRNAs into cells (Sledz *et al.*, 2003). siRNAs targeting Lamin A/C and GAPDH were transfected into a human glioblastoma line. They observed a siRNA dose-dependent decrease in the target gene (as expected) but there was also an increase in the expression of interferon-stimulated genes. Their studies showed that the actual RNAi mechanism is independent of the interferon system.

The processing of long dsRNA into siRNA by Dicer and the unwinding of the siRNA duplexes in RISC to form an active complex has been shown to require ATP in

Drosophila embryo lysates (Bernstein *et al.*, 2001; Nykanen *et al.*, 2001). Only siRNA with a 5' phosphate can cause RNAi in *Drosophila*, the addition of phosphate to the 5' end requires ATP (Nykanen *et al.*, 2001). ATP was not required for Dicer activity in mouse EC cells (Billy *et al.*, 2001) or by recombinant human Dicer (Provost *et al.*, 2002; Zhang *et al.*, 2002). ATP has since been shown not to be required for mRNA cleavage but in *Drosophila* RISC is 'turned over' more rapidly in its presence. Therefore ATP either allows quicker product release or promotes a conformational change in RISC which allows it to bind to other mRNA molecules (Haley and Zamore, 2004).

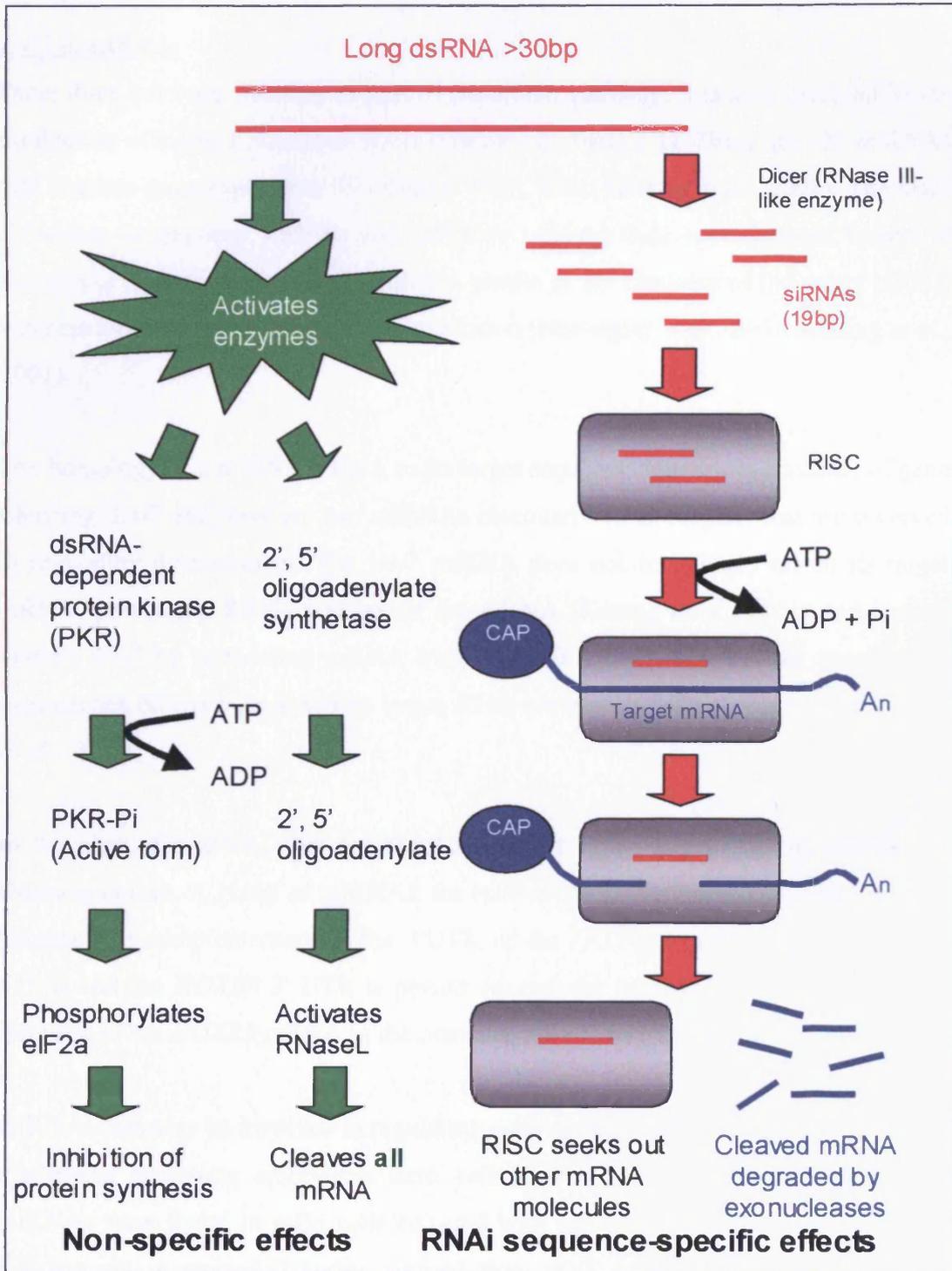


Figure 1.1. Overview of the mechanism of RNA interference. DsRNA (>30bp) when introduced into mammalian cells can follow two pathways. 1) **Specific pathway-** DsRNA is cleaved into smaller ~21 nucleotide fragments (siRNAs) by an RNase III enzyme Dicer. siRNA duplexes then act in conjunction with another endonuclease enzyme complex, the RNA-induced silencing complex (RISC) resulting in specific cleavage of the target RNA sequence. 2) **Non-specific pathway-** dsRNA (>30bp) activates two enzymes: PKR which phosphorylates translation initiation factor eIF2a, inhibiting protein synthesis; 2' 5' oligoadenylate synthetase synthesises a molecule that activates RNaseL, which targets all mRNAs for degradation.

1.1.1.a. miRNAs

Dicer does not only function as part of the RNAi pathway, it is also involved in the production of micro RNAs (miRNAs) (Figure 1.2, Table 1.1). These are ~21 nt RNAs that regulate gene expression (Hutvagner *et al.*, 2001; Ketting *et al.*, 2001). The main difference in the way siRNAs and miRNAs regulate their specific gene targets in metazoans is that introduction of siRNAs results in the cleavage of the target mRNA whereas most miRNAs inhibit RNA translation (Hutvagner *et al.*, 2001; Ketting *et al.*, 2001).

The homology of a miRNA/siRNA to its target sequence determines its mode of gene silencing. *let-7* and *lin-4* are two miRNAs discovered in *C. elegans* that are involved in regulating development. The *let-7* miRNA does not completely match its target mRNA, preventing RISC cleavage of the mRNA (Ketting *et al.*, 2001) and instead inhibits *let-7* by preventing mRNA translation. It was shown that the presence of mismatches between *lin-4* and its target RNA are essential for proper regulation (Ha *et al.*, 1996).

In the case of miRNAs that are exact matches to their target mRNA, siRNA-like cleavage occurs. A group of miRNAs, the *miR-196*, found conserved in fish, frog and humans, are complementary to the 3'UTR of the *HOX* genes. Pairing between *mi-R196a* and the *HOXB8* 3' UTR is perfect (except for one G:U pairing) resulting in cleavage of the *HOXB8* mRNA in the presence *mi-R196a* (Yekta *et al.*, 2004).

miRNAs that may be involved in regulating early mammalian development have been discovered in mouse embryonic stem cells (mES) (Houbaviy *et al.*, 2003). Six miRNAs were found in mES cells cultured with and without feeders in presence of LIF but not in embryoid bodies formed from mES cells cultured for 14 days in suspension. Therefore it is predicted these miRNAs have a role in maintaining the pluripotency of stem cells.

It has been estimated using, computer analysis, that there are 200-250 human miRNAs (Lim *et al.*, 2003a). Lu and colleagues looked at the expression patterns of miRNAs and found the expression levels of most miRNAs are reduced in human tumours when compared to normal tissue (Lu *et al.*, 2005). Also the pattern of

miRNA expression in tumours was shown to be related to the severity and origin of the tumour. Therefore the study showed the potential of miRNA profiles for classification of human cancers.

miRNAs	siRNAs
~22 nucleotide (nt) RNAs	
Can induce transcriptional silencing by DNA methylation	
In mammalian cells and <i>C. elegans</i> are processed by a single Dicer	
Synthesised from endogenous ~70nt transcripts that form hairpin structures.	Formed from dsRNA.
In <i>Drosophila</i> produced by Dcr-1	In <i>Drosophila</i> produced by Dcr-2
After processing miRNA is made up of two strands, one from each arm of the hairpin RNA.	Processing of dsRNA results in production of siRNAs of different sequences.
miRNA sequence usually only partially complementary to target RNA, often targets the 3' UTR.	siRNAs are normally homologous to the target mRNA sequence to which they bind.
Sequences of miRNAs are generally conserved between species.	siRNAs show less conservation of sequence.
Most regulate gene expression by inhibiting mRNA translation.	Reduces gene expression of target gene by degradation of mRNA.

Table 1.1. Comparison of the structures of miRNAs and siRNAs [Information taken from (He and Hannon, 2004; Hutvagner and Zamore, 2002; Lee *et al.*, 2004; Lim *et al.*, 2003b; Tomari and Zamore, 2005)].

miRNAs are transcribed as long primary transcripts (pri-miRNAs) which are then cleaved to shorter 70 nt stem loop precursors (pre-miRNAs) in the nucleus by RNase III enzyme Drosha in humans (Lee *et al.*, 2003) (Figure 1.2). This process is thought to be conserved in animals as Drosha homologues have been found in *C. elegans*, *D. melanogaster* and mice (Filippov *et al.*, 2000; Fortin *et al.*, 2002). These pre-miRNAs are transported out of the nucleus by Exportin 5 (Lund *et al.*, 2004) and subsequently cleaved by a second RNase III, Dicer, in the cytoplasm into mature miRNAs (Lee *et al.*, 2002).

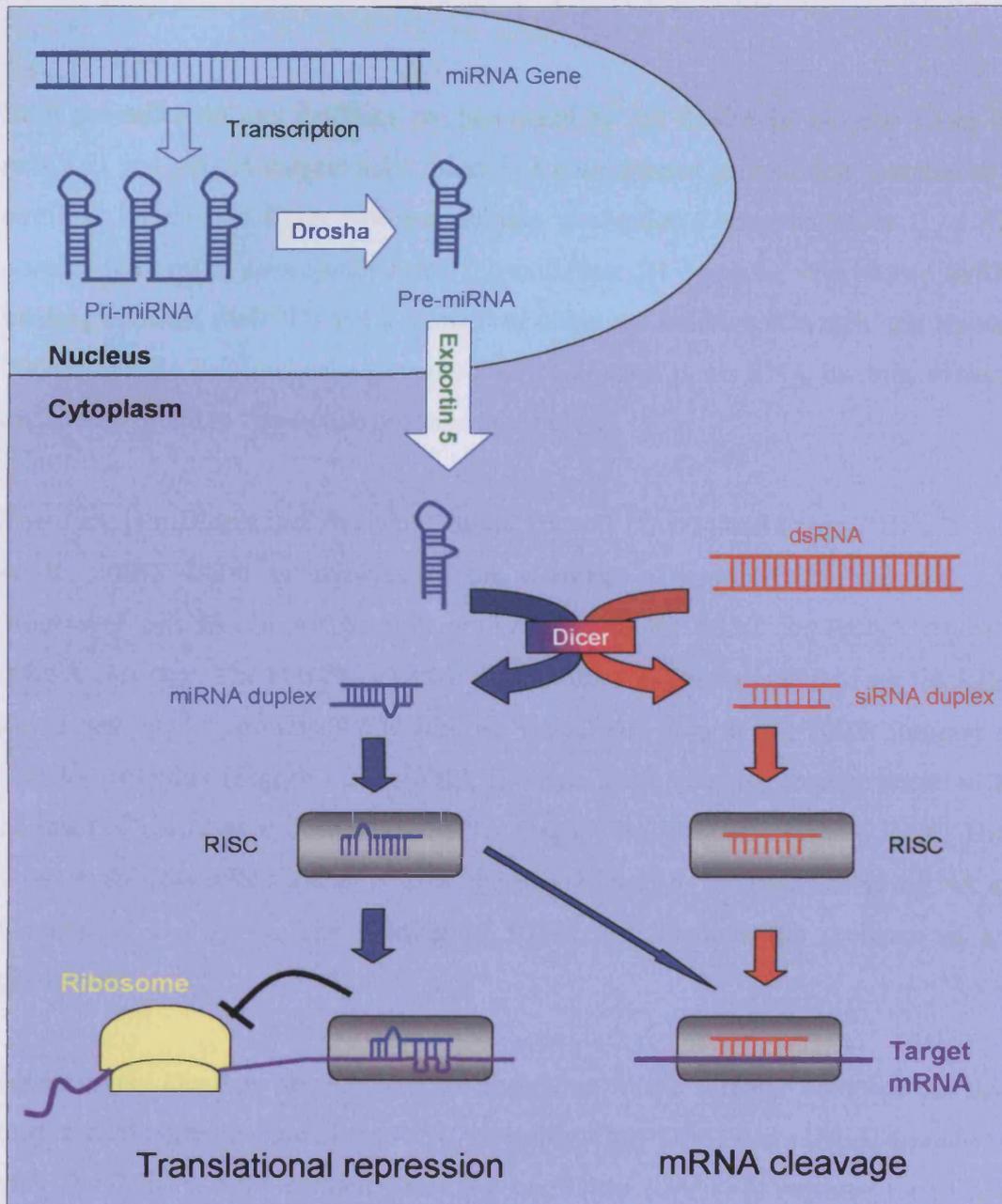


Figure 1.2. Mechanism by which miRNAs and siRNAs suppress gene expression. miRNAs are transcribed as long primary transcripts (pri-miRNAs) that are subsequently cleaved by RNase III enzyme Drosha to 70 nt stem loop precursors (pre-miRNAs). Exportin 5 transports the pre-miRNAs to the cytoplasm where they are cleaved by a second RNase III enzyme Dicer into mature miRNAs. siRNA duplexes are formed from dsRNA by cleavage with Dicer. A single strand of the miRNA or siRNA is incorporated into RISC. The RISC:miRNA/siRNA complex then binds to its target sequence resulting in mRNA cleavage or translational repression. [Figure adapted from (He and Hannon, 2004).]

1.1.1.b. Dicer

Both pre-miRNAs and dsRNAs are processed by the RNase III enzyme Dicer into miRNAs and siRNA respectively. Dicer is a multidomain protein that contains an N-terminal DExH-box RNA helicase domain (exception *Drosophila* Dcr-1), a PAZ domain (exception *Drosophila* Dcr-2), two RNase III domains, one or two dsRNA binding domains (dsRBD) and a domain of unknown function (Carmell and Hannon, 2004). The PAZ (pinwheel-argonaute-zwille) domain is an RNA binding molecule and is also found in Argonaute proteins (see below).

There are two Dicers in *Drosophila* called Dicer-1 (Dcr-1) and Dicer-2 (Dcr-2) (Lee *et al.*, 2004). Dcr-1 is involved in the cleavage of pre-miRNAs and Dcr-2 for processing dsRNAs, although both are required in the RISC for siRNA mediated mRNA cleavage. The two Dicers have distinct roles in the formation of siRNA:RISC. Dcr-2 and another protein R2D2 bind to the siRNA (Liu *et al.*, 2003) forming the initiator complex (Figure 1.3). siRNA induced gene silencing cannot occur in the absence of R2D2 as it is required for loading of the siRNA:Dcr2 onto RISC. Dicer binds to the less stable end of siRNA and R2D2 binds to the more stable siRNA end (Tomari *et al.*, 2004). The binding of R2D2 also requires the presence of a 5' phosphate.

Alternatively Dcr-1 is not required for formation of the initiator complex but helps form a stable intermediate of the RISC assembly. Only Dcr-1 has a DExH domain and only Dcr-2 has a PAZ domain. Only the one Dicer (Dcr-1) is required for miRNA silencing. Dcr-1 associates with Loquacious (Loqs), a paralog of R2D2; mutation of Loqs in *Drosophila* prevents the normal processing of pre-miRNAs (Forstemann *et al.*, 2005).

There are only single forms of Dicer found in *C. elegans*, mice and humans. In *C. elegans* mutations in *dcr-1* show that it has an important role in germ-line development (Knight and Bass, 2001). Ketting and colleagues showed that the phenotype seen in loss of function mutations of the *dcr-1* gene in *C. elegans* was the same as that seen in knock-down of the *let-7* gene (Ketting *et al.*, 2001). Subsequently they showed that Dcr-1 is involved in the processing of ds *let-7* RNA

into the 21 nt active siRNA. RDE-4 protein interacts with Dcr-1 as well as members of RISC including RDE-1. It has two copies of the dsRNA binding motif (dsRBM) and functions to present the dsRNA for Dicer processing (Tabara *et al.*, 2002). RDE-1 does not appear to be involved in miRNA induced gene silencing.

It has been shown that Dicer is needed for normal development and stem cell maintenance in the mouse embryo (Bernstein *et al.*, 2003). Dcr-1 deficient mice are not viable, embryos examined at embryonic day 7.5 failed to express the primitive streak marker, Brachyury and were morphologically abnormal (Bernstein *et al.*, 2003). A DCR-1 deficient mES cell line was produced by conditional gene targeting (Kanellopoulou *et al.*, 2005). The cells were viable even though they were unable to process miRNAs or perform RNAi. The DCR-1 deficient mES cells formed embryoid bodies but did not show the normal expression patterns of mES cell genes observed upon differentiation. For example levels of *Oct-4* were found to remain constant on the formation of EB whereas normally *Oct-4* is downregulated upon differentiation.

1.1.1.c. RISC

The RNA-induced silencing complex (RISC) is a multimeric protein that complexes with Dicer produced siRNAs/miRNAs and targets mRNA, resulting in cleavage of the mRNA or inhibition of translation. RISC contains Argonaute proteins and it was proposed that the presence of different Argonaute proteins in RISC assist in specifying by which mechanism the siRNAs or miRNAs execute gene silencing (Okamura *et al.*, 2004).

Argonaute proteins are a highly conserved family of ~100kD proteins that have an N-terminal PAZ domain and a C-terminal PIWI domain (Carmell *et al.*, 2002; Cerutti *et al.*, 2000) (Table 1.2). The PAZ domain is an RNA binding module that recognises the 2 nt 3' overhangs characteristic of siRNAs (Lingel *et al.*, 2004). Part of the human PIWI domain has been shown to be involved in protein-protein interactions between Argonaute and Dicer (Doi *et al.*, 2003) possibly for enhanced siRNA transfer in RISC. The PIWI domain has structural homology with the endoribonuclease RNase H, RNase H cleaves the RNA strand of a DNA-RNA hybrid (Parker *et al.*, 2004). PIWI domains of Argonaute proteins have been proposed to be the endonuclease

subunit of RISC sometimes referred to as 'slicer' (Liu *et al.*, 2004; Parker *et al.*, 2004).

There are four characterised Argonaute proteins in *Drosophila* including Argonaute 1 (Ago1) and Argonaute 2 (Ago2) (Carmell *et al.*, 2002). Ago1 was shown to be specific for processing of miRNA and is not required for siRNA induced gene silencing. Conversely Ago2 mutants are defective in siRNA silencing but process miRNAs as normal (Okamura *et al.*, 2004).

Other proteins found to be associated with *Drosophila* RISC include the vasa intronic gene (VIG) protein and the *Drosophila* homolog of the fragile-X mental-retardation protein called dFXR (Ishizuka *et al.*, 2002) (also known as dFMR1). They both have RNA-binding domains but their function in RISC are undetermined (Caudy *et al.*, 2002). *Drosophila* RISC has also been shown to contain tudor-staphylococcal nuclease (TSN).

In *C. elegans* the members of the Argonaute family involved in gene silencing are RDE-1 (Tabara *et al.*, 1999), PPW-1, (Tijsterman *et al.*, 2002) Alg-1 and Alg-2 (Grishok *et al.*, 2001). RDE-1 is required for siRNA induced gene silencing (Grishok *et al.*, 2000) with Alg-1 and Alg-2 required for the production of mature miRNAs (Grishok *et al.*, 2001).

Argonaute proteins in humans and mice include eIF2C1 (AGO1), eIF2C2 (AGO2), eIF2C3 and eIF2C4 and were shown to interact with Dicer but only eIF2C1 was found to be essential for siRNA processing (Doi *et al.*, 2003; Liu *et al.*, 2004).

In order for an siRNA to induce RNAi it must have a 5' phosphate (Schwarz *et al.*, 2002). It was also predicted that a single strand of siRNA duplex is incorporated into RISC. Transfection of antisense strands of siRNA of same sequence results in gene specific knock-down using the same pathway as RNAi, although this was shown to be >10-fold less effective than using siRNA duplexes. Khvorova and colleagues looked at the internal stability of siRNAs and miRNA and suggested that this is critical in deciding whether the sense or the antisense strand is retained by RISC (Khvorova *et al.*, 2003). They found that functional siRNAs and miRNAs had enhanced flexibility

at the antisense 5' terminus and an internal stability profile that is generally low but especially within 9-14 bp region (starting from 5' antisense end) of the miRNA/siRNA duplex. The authors suggest the low stability in the centre of the region homologous to the target mRNA may allow release of the cleaved mRNA quickly from the RISC complex. This allows the active RISC to seek new mRNA target or it may enable RISC to function properly by allowing the correct conformation of the complex to form.

Schwarz and colleagues also predicted that only one strand of the siRNA duplex is incorporated into RISC and the other strand is degraded (Schwarz *et al.*, 2003). The strand remaining part of the active RISC is the strand with the lowest binding energy to its complement at its 5' end. They concluded that when designing shRNAs a mismatch or G:U base pair should be placed at the 5' of the antisense strand. Promoting retention of the correct strand in the active RISC and it also reduces the chance of non-sequence specific off-target effects to the shRNA.

In human cells RNAi occurs in the cytoplasm but not in the nucleus although, during the process of mRNA export, RNAi can occur (Zeng and Cullen, 2002). Subsequently Argonaute 2/RISC complexes were found to be localised to cytoplasmic bodies in human embryonal kidney 293 cells (Sen and Blau, 2005). These cytoplasmic bodies had previously been shown to be the site of mRNA decay, containing exonucleases and decapping enzymes (Cougot *et al.*, 2004). Therefore localisation of the AGO2/RISC to these cytoplasmic bodies should allow rapid degradation of mRNAs that have been cleaved by RISC. AGO1 was also shown to be localised to the cytoplasmic body.

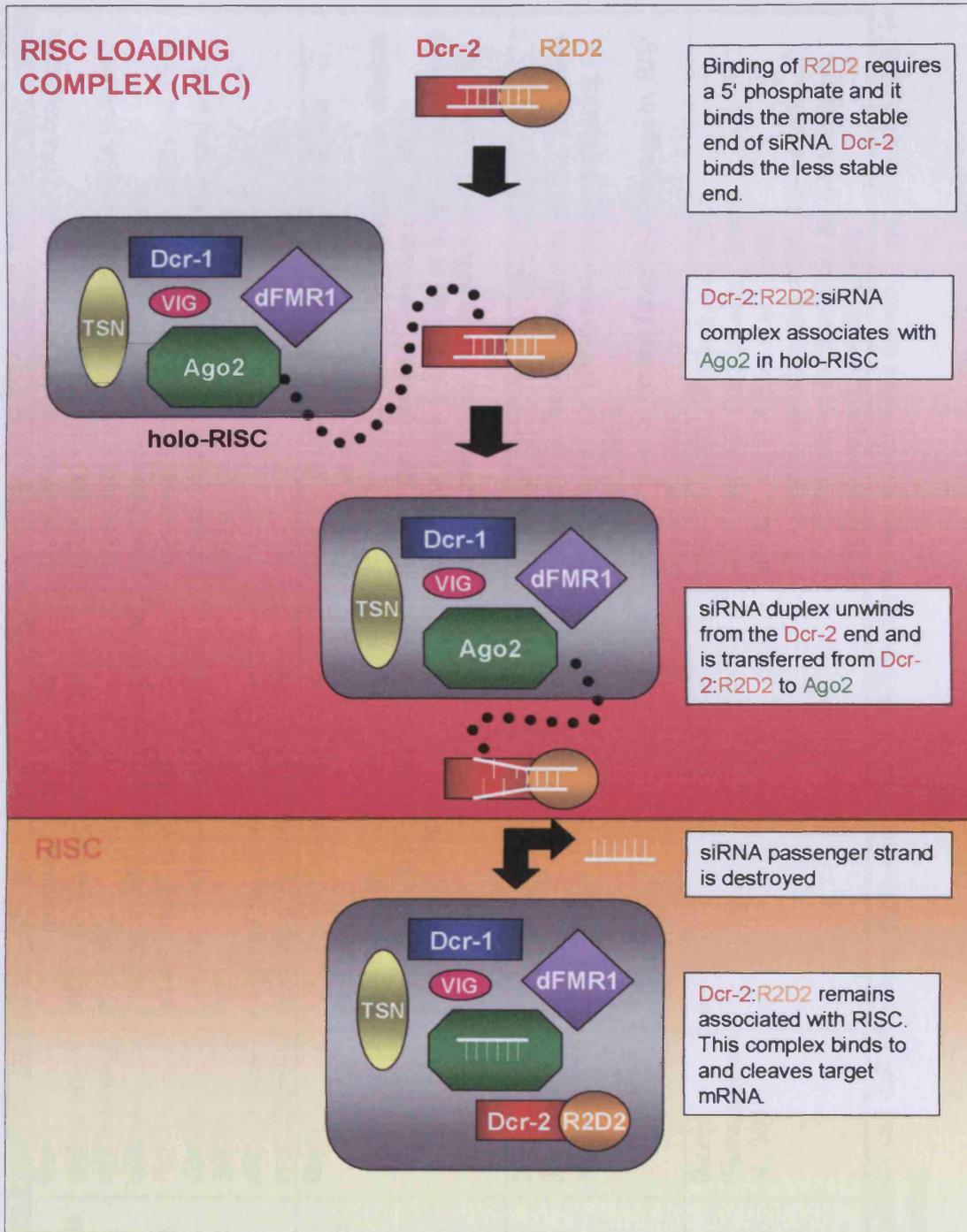


Figure 1.3. Assembly of siRNAs into RISC in *Drosophila*. siRNA is bound by R2D2 at its more stable end and Dcr-2 at its less stable end. The Dcr-2:R2D2:siRNA complex associates with other proteins to form the RISC loading complex. This complex includes the vasa intronic gene (VIG) protein, *Drosophila* homolog of the fragile-X mental-retardation protein called dFXR (also known as dFMR1) and TSN (tudor-staphylococcal nuclease) whose functions are unknown. The siRNA unwinds in an ATP-dependent process and one strand associates with the PAZ domain of Ago-2. The other strand is destroyed. The Dcr:R2D2 remains associated with the RISC complex that is now ready to cleave its target mRNA. [Figure adapted from Tomari and Zamore (Tomari and Zamore, 2005)].

Organism	miRNA processing	Dicer	RISC Loading complex	Argonaute proteins
<i>Drosophila</i>	Drosha - cleaves pri-miRNA to pre-miRNA in nucleus. Pasha - assists Drosha and is a dsRNA binding protein (dsRBD) (Denli <i>et al.</i> , 2004).	Dcr-1 - cleaves pre-miRNAs to miRNAs Dcr-2 - cleaves dsRNA to siRNAs (Lee <i>et al.</i> , 2004)	R2D2 - associates with Dcr-2 and siRNA for loading onto RISC (Liu <i>et al.</i> , 2003). - without R2D2 Dicer produced siRNAs are not loaded onto RISC. Loquacious (Loqs) - associates with Dcr-1. - required for normal pre-miRNA processing (Forstemann <i>et al.</i> , 2005).	Argonaute1 (Ago1) - involved in miRNAs biogenesis, (Okamura <i>et al.</i> , 2004). Argonaute 2 (Ago2) - required for siRNA induced gene silencing (Okamura <i>et al.</i> , 2004). Aubergine (Aub) (Kennerdell <i>et al.</i> , 2002) and Piwi are also involved in RNA silencing (Carmell <i>et al.</i> , 2002).
<i>C. elegans</i>	Drosha (DRSH-1) Pasha (Pash1) (Denli <i>et al.</i> , 2004).	Dcr-1	RDE-4 - only required for siRNA processing - interacts with dsRNA, Dcr-1 , DExH-box helicase (DRH-1) and RDE-1 (Tabara <i>et al.</i> , 2002).	RDE-1 - initiates RNAi in response to dsRNAs (Grishok <i>et al.</i> , 2000). PPW-1 (= homolog of RDE-1) - required for efficient RNAi in the germline (Tijsterman <i>et al.</i> , 2002). Alg-1 and Alg-2 (=homologs of RDE-1) - required for the accumulation of mature miRNAs (Grishok <i>et al.</i> , 2001).
Mice	Drosha (Fortin <i>et al.</i> , 2002).	Dcr-1 (Bernstein <i>et al.</i> , 2003)	----	eIF2C1, eIF2C2, eIF2C3, eIF2C4 (AGO 1-4) all associate with Dicer although only eIF2C1 was found to be essential for siRNA mediated gene silencing (Doi <i>et al.</i> , 2003). AGO2 - must be present for mRNA cleavage by RISC (Liu <i>et al.</i> , 2004).
Humans	Drosha (Lee <i>et al.</i> , 2003). DGCR8 - is a homolog of pasha (Gregory <i>et al.</i> , 2004; Han <i>et al.</i> , 2004).	Dicer (Hutvagner <i>et al.</i> , 2001)	----	eIF2C1/AGO1 (Martinez <i>et al.</i> , 2002) eIF2C2/AGO2 (Meister <i>et al.</i> , 2004) eIF2C3, eIF2C4 (Doi <i>et al.</i> , 2003) All 4 proteins associate with RISC. The presence of AGO2 was shown to be essential for RNAi induced mRNA cleavage (Liu <i>et al.</i> , 2004).

Table 1.2. Proteins involved in pri-miRNA and dsRNA processing to miRNAs and siRNAs respectively and proteins known to associate with RISC in *C. elegans*, *Drosophila*, mice and humans.

1.1.2. Previous Studies

Since RNAi was discovered in *C. elegans* (Fire *et al.*, 1998) it has been utilised to study gene expression in many different species including *Drosophila*, mouse and human cells using dsRNA, shRNAs and siRNAs produced in different ways (Table 1.3). Transfection of siRNA duplexes or dsRNA only allows transient knock-down of gene expression (Holen *et al.*, 2002; Kennerdell and Carthew, 2000; Yang *et al.*, 2001). Stable expression of dsRNA hairpins in cells enables expression of the gene of interest to be suppressed for longer. Several vectors have been designed that either express hairpin dsRNAs or hairpin siRNAs (shRNA).

RNAi inducer	Method of production	Reference
dsRNA	T7 polymerase produced.	(Yang <i>et al.</i> , 2001)
	Vectors expressing sense and antisense RNA under tetracycline control.	(Wang <i>et al.</i> , 2003)
	Bacteria expressing dsRNA fed to <i>C. elegans</i> .	(Kamath <i>et al.</i> , 2001) (Timmons <i>et al.</i> , 2001)
siRNAs	Chemically synthesised.	(Harborth <i>et al.</i> , 2001) (Hay <i>et al.</i> , 2004)
	T7 polymerase produced.	(Donze and Picard, 2002)
	Recombinant Dicer produced pools from dsRNA.	(Kawasaki <i>et al.</i> , 2003)
	Vectors expressing single sense and antisense siRNAs each using a U6 promoter.	(Miyagishi and Taira, 2002)
shRNA	RNA III polymerase promoter (H1/U6) containing vectors.	(Brummelkamp <i>et al.</i> , 2002; Paul <i>et al.</i> , 2002; Sui <i>et al.</i> , 2002; Velkey and O'Shea, 2003)
	RNA II polymerase promoter of U1 snRNA containing vector.	(Denti <i>et al.</i> , 2004)
	human 7S K promoter (also a RNA polymerase III promoter) containing vector.	(Koper-Emde <i>et al.</i> , 2004)
	retroviral vectors.	(Barton and Medzhitov, 2002; Devroe and Silver, 2002)
	lentiviral-based vectors.	(An <i>et al.</i> , 2003; Rubinson <i>et al.</i> , 2003; Zaehres <i>et al.</i> , 2005).

Table 1.3. Different methods used to induce RNAi in cells and organisms.

1.1.2.a. RNAi in non-mammalian cells

The discovery of RNAi in metazoans was made during a study by Fire and colleagues (Fire *et al.*, 1998). They noted that introduction of dsRNA (between 300 and 1,030 base pairs) into *C. elegans* reduced the expression of genes more effectively than either of the sense or antisense strand of RNA. It was later found that these dsRNAs were processed to shorter RNA duplexes of about 25 nt in *Drosophila* (Hammond *et al.*, 2000). Elbashir and colleagues showed that chemically synthesised 21 and 22 nt siRNA duplexes reduced gene expression specifically in *Drosophila* lysate (Elbashir *et al.*, 2001b).

siRNA duplexes were designed to be 21 nt long as studies on processing of dsRNA into siRNA duplexes in *Drosophila* found 21 nt siRNAs to be the most common (45%), with the next most abundant were 22 nt long (28%) (Elbashir *et al.*, 2001b). siRNA duplexes have also been shown to specifically knock-down gene expression in *C. elegans* (Caplen *et al.*, 2001) and mammalian cells (Elbashir *et al.*, 2001a; Harborth *et al.*, 2001).

In *Drosophila*, integration of a construct that transcribed a hairpin-looped dsRNA resulted in specific gene silencing (Piccin *et al.*, 2001). This construct contained two inverted repeats of part of the target gene sequence separated by a 330bp spacer sequence. The spacer was included to increase the efficiency of cloning in *Escherichia coli*. Spacers up to a third of the inverted repeat length increased cloning efficiency in *E. coli* and did not reduce the capacity of the dsRNA to perform RNAi.

Paddison and colleagues looked at RNAi induced gene silencing in *Drosophila* S2 cells using short hairpin RNAs (shRNAs) (Paddison *et al.*, 2002). They found using different loop sizes (from 4 to 23 bases) did not affect the silencing efficiency of the target gene. They also showed these shRNAs could knock-down gene expression in human embryonic kidney cells. They used 29 nt shRNA duplexes although it was noted that duplexes of 25 nt could be used with little loss of RNAi. T7 RNA polymerase was used to synthesise shRNAs *in vitro*.

Fraser and colleagues used RNAi to study the function of uncharacterised genes in *C. elegans* chromosome I by feeding worms bacteria expressing dsRNA, each targeting a

single gene (Fraser *et al.*, 2000). From their study they were able to determine the function of 13.9% of the genes investigated.

A larger library was subsequently constructed that knocked down ~86% of *C. elegans*' 19,427 predicted genes (Kamath and Ahringer, 2003). These bacteria were fed to *C. elegans* and phenotypes of the nematodes were studied. 1,722 dsRNAs that caused sterility, embryonic or larval death, slow growth or defects by RNAi gene silencing were identified. Confirmation of the effectiveness of this RNAi screen was shown by comparing the RNAi produced phenotypes to known loss of function models and finding that they corresponded in 92% of cases.

Other studies utilising the same library identified genes that protect *C. elegans* from mutations (Pothof *et al.*, 2003) or genes involved in apoptosis (Lettre *et al.*, 2004). The first of the aforementioned studies identified 61 genes that were predicted to have roles in control of cell cycle, DNA repair, replication and chromatin organisation. 82% of the genes identified have human orthologs and it was suggested that some of these genes are new oncogene candidates. In the second study 21 genes were identified that, when knocked down by RNAi, increased germ cell death.

1.1.2.b. RNAi in mammalian cells

The use of RNAi in mammalian cells was originally thought to be limited to undifferentiated embryonic stem (ES) cells, embryonal carcinoma (EC) cells, early embryos and oocytes that do not have an interferon response (Billy *et al.*, 2001; Caplen *et al.*, 2000; Svoboda *et al.*, 2000; Wianny and Zernicka-Goetz, 2000). Specific reduction in gene expression was observed when dsRNA targeting Mos and the tissue plasminogen activator (tPA) was injected into mouse oocytes (Svoboda *et al.*, 2000). In another study injection of dsRNA reduced the expression of a constitutively expressed green fluorescent protein (MmGFP) in mouse embryos (Wianny and Zernicka-Goetz, 2000). After injection of MmGFP dsRNA into transgenic blastocysts a reduction in GFP was observed that remained up until 6.5 days postimplantation.

In later studies hairpin dsRNA or hairpin RNA expressing constructs were injected into mouse oocytes and preimplantation embryos and were shown to be as effective at

induction of RNAi as dsRNA (Svoboda *et al.*, 2001). The vector constructed contained an inverted repeat of 535 bp of the target gene with a spacer sequence. Inclusion of the short spacer (20-50 bp) between the repeat greatly increased inverted-repeat cloning efficiency.

Induction of RNAi in mouse embryonal carcinoma cell lines and mES cells grown in culture using dsRNA has also been shown. Billy and colleagues saw a reduction (between 60-90%) in expression of reporter genes encoding GFP and β -galactosidase by transfection of 700bp dsRNA (Billy *et al.*, 2001). Also a specific reduction in expression of the target gene using hairpin dsRNA has been observed in mES cells (Yang *et al.*, 2001). GFP was targeted and a vector was constructed expressing an inverted repeat of the GFP gene (547 nt). Then either the hairpin dsRNA expressing vector or dsRNA was transfected into mES cells. A specific reduction in GFP was observed in transfected cells but was found to decrease over time. This was probably due to increase in mES cell number and subsequent reduction in the amount of dsRNA per cell.

Transfection of dsRNA into somatic mammalian cells was shown to result in non-specific mRNA degradation or no response at all (Caplen *et al.*, 2000). Discovery of siRNAs as intermediates in RNAi mechanism (Elbashir *et al.*, 2001a; Elbashir *et al.*, 2001b) that do not trigger an interferon response led to the development of RNAi techniques for gene expression studies in mammalian cell culture (Brummelkamp *et al.*, 2002; Caplen *et al.*, 2001; Devroe and Silver, 2002; Leirdal and Sioud, 2002; Yu *et al.*, 2002).

The reduction in gene expression by transfection of siRNA duplexes in mammalian cell lines including human HeLa cells, SV40-transformed rat fibroblasts and mouse 3T3 cells were studied (Harborth *et al.*, 2001). Many genes were used as targets for RNAi including seven that had been previously characterised using other methods. Reduction in gene expression using RNAi confirmed the findings of previous studies with regard to the function of these genes.

Vectors that express short hairpin (sh) RNA duplexes targeted to a gene of interest in mammalian cells have been constructed, many utilising the polymerase III promoters

H1, U6 small nuclear RNA (snRNA) and 7S K RNA gene promoter (Brummelkamp *et al.*, 2002; Koper-Emde *et al.*, 2004; Sui *et al.*, 2002; Tang *et al.*, 2004). This type of promoter was chosen because the RNA it synthesises has no polyadenosine tail, a definite start site and termination signal of transcription consisting of four or five consecutive thymidines. Also the cleavage of this RNA occurs after the second uridine when reaching the termination site resulting in the production of shRNAs that have the characteristic overhanging ends as observed in siRNAs (Elbashir *et al.*, 2001b). Brummelkamp and colleagues successfully knocked-down the expression of more than ten genes by electroporation of such a vector into MCF-7 cells (human breast cancer cell line) (Brummelkamp *et al.*, 2002). shRNAs were produced from an inverted repeat of 19 nt of target gene transcript separated by a 'spacer' sequence (5-9 nt) that forms the hairpin loop under the control of the H1 RNA polymerase III promoter. The knockdown observed by transfection of the construct expressing shRNA was comparable to that of the corresponding chemically synthesised siRNA.

The mouse RNA polymerase III U6 promoter was used to knockdown the reporter gene GFP as well as the endogenously expressed Lamin A/C (*LMNA*) and cyclin dependent kinase 2 (*CDK2*) in 4 cell lines (HeLa, H1299, C-33A, U-2 OS) (Sui *et al.*, 2002). An inverted repeat matching 21 nt of the target gene with a 6 nt spacer was cloned into a vector containing the U6 promoter. Transfection of the vectors resulted in knockdown of the target proteins. LMNA and CDK-2 negative cells made up between 0.2% and 5.2% of the control cells compared to between 86.9 and 95.2% in shRNA plasmid transfected cells.

Different siRNAs/shRNAs targeting the same gene have been shown to have different efficiencies at knocking down the target gene (Harborth *et al.*, 2001; Hemann *et al.*, 2003; Holen *et al.*, 2002). This could possibly be due to the accessibility of target site within the mRNA to allow base pairing with the siRNA (Scherr *et al.*, 2003) or due to the 'wrong strand' (sense strand) being loaded into RISC. It was found that the internal stability of siRNAs and miRNA is critical in deciding whether the sense or the antisense strand is retained by RISC (Khvorova *et al.*, 2003).

Targeting of the *Trp53* tumour suppressor gene encoding p53 in hematopoietic stem cells using three different shRNAs resulted in distinct *in vivo* phenotypes when these

cells were transplanted into lethal irradiated recipient mice (Hemann *et al.*, 2003). The level and severity of the tumour correlated with the amount of p53 knockdown produced by the individual shRNA by RNAi. The amount of knockdown also correlated with the previously identified levels *in vitro*.

Retroviral vectors allow the delivery of shRNA expression cassettes into cells that are difficult to transfect, such as primary cells. Also, as the retrovirus integrates into the host genome, it allows stable cell lines expressing the shRNA of interest to be produced. Studies have demonstrated the potential of retroviral-based delivery of shRNA. In one study a retroviral vector was designed to express shRNA from the human H1 promoter. The shRNA targeted p53 and was used to infect HEK 293T cells and primary human fibroblasts inducing specific knockdown of p53 (Barton and Medzhitov, 2002). In a second study a retroviral vector was constructed this time with the expression cassette containing U6 promoter and the shRNA (Devroe and Silver, 2002). This vector was also shown to induce sequence specific knockdown by RNAi.

Lentiviral vectors have also been designed to express shRNA. The advantage of these vectors is that they infect cells at high efficiency and will also infect non-dividing cells. An and colleagues cloned the human U6 polymerase III transcriptional unit into a self-inactivating lentiviral vector that expresses GFP (An *et al.*, 2003). They cloned shRNA to the humanized firefly luciferase to test the vector by cotransfecting it together with a vector expressing the Luciferase into human 293T cells. Their vector was found to reduce the firefly Luciferase 10-fold as compared to the control cells.

Zaehres and colleagues knocked down a GFP transgene and the endogenous genes of Nanog and Oct-4 using a lentiviral vector in hES cells (Zaehres *et al.*, 2005). RNAi induced knockdown of *Nanog* resulted in a reduction in the expression of *Oct-4*. Likewise there was a reduction in *Nanog* when *Oct-4* was knocked down. This suggested Nanog and Oct-4 are co-regulated in hES cells.

1.1.3. Present and potential applications of RNAi

Advantages of using RNAi over current techniques for gene silencing are that it is more reliable, works more often and reduces the level of gene expression of the target

to lower levels than seen with antisense techniques, (Caplen *et al.*, 2001). RNAi has been used for genome-wide study of gene function (Fraser *et al.*, 2000), identifying cancer related genes (Williams *et al.*, 2003) and for the production of transgenic mice (Carmell *et al.*, 2003). The potential of RNAi to treat viral infection (Capodici *et al.*, 2002; Gitlin *et al.*, 2002; McCaffrey *et al.*, 2003), heart disease (Soutschek *et al.*, 2004) and improve cancer therapy (Yague *et al.*, 2004) has also been shown.

1.1.3.a. RNAi Libraries

The development of siRNA and shRNA expression libraries has allowed large scale gene specific silencing to determine the functions of genes. There are three types of screening procedures used with RNAi libraries at present (Silva *et al.*, 2004). The first is transfection of siRNAs or shRNA expressing plasmids into cells seeded in multiwell plates. Each well is transfected with one siRNA/shRNAs targeting a single mRNA. Plates are screened for cells increasing or decreasing the expression of a reporter gene, for example GFP, by observing cells or conducting biochemical assays on the cells. The second way that RNAi libraries may be used is that pools of vectors expressing dsRNA may be transfected into cells followed by isolation of individual colonies. The phenotypes of these colonies can be scored, for example for a change in morphology. Alternatively 'reverse transfections' can be performed. Cells are seeded onto a glass slide and then transfection mix containing different siRNAs/shRNAs are spotted onto the cells in defined places. Selection is applied to the cells and the resulting colonies are scored. The third screening procedure used with RNAi libraries is transfection of the whole library into cells and selection of the cells. Each shRNA transfected contains a unique DNA barcode that can be 'read' by hybridising the pool of isolated shRNAs after screening to an oligonucleotide microarray that has all of the unique sequences spotted on it. The profile of the shRNAs abundance can be compared to controls to identify genes involved in a particular pathway or process. Examples of the way in which these different libraries have been used will be described below.

A library was constructed that contained bacteria expressing dsRNAs that target ~86% of *C. elegans*' 19,427 predicted genes (Kamath and Ahringer, 2003). These bacteria were fed to *C. elegans* and phenotypes of the nematodes were studied. 1,722 dsRNAs that caused sterility, embryonic or larval death, slow growth or defects by

RNAi gene silencing were identified. Confirmation of the effectiveness of this RNAi screen was shown by comparing the RNAi produced phenotypes to known loss of function models and finding that they corresponded in 92% of cases.

Paddison and colleagues generated a shRNA expression library targeting 9,610 human and 5,563 mouse genes (Paddison *et al.*, 2004). Vector hairpin design included a 27 nt U6 leader sequence followed by 29bp of dsRNA with a 4 nt loop. The library can either be used by genetic selection or for screening. Selections conferring growth advantage on cells can be performed after transfection of pooled shRNAs. Genetic screening, for example for cell death, is labour intensive and time consuming, as the cells are seeded into 96-well plates, with each well transfected with an individual construct. Therefore a 'DNA bar coding strategy' was used. A unique 60 nt sequence was included in each shRNA vector. The relative levels of shRNAs were determined by hybridisation to a microarray containing 60 nt oligonucleotides each complementary to an individual shRNA vector.

In another study a shRNA retroviral vector library was constructed targeting 7,914 different human genes with 3 different shRNAs for each gene (Berns *et al.*, 2004). The library included shRNAs against genes involved in regulation of transcription, cell cycle signalling as well as those genes predicted to be involved in disease. The library was used to study the p53 pathway and identified five genes whose suppression resulted in resistance of cells to p53 arrest.

In this study a temperature-sensitive human BJ fibroblast cell line was constructed that proliferated at 32°C but upon shift to 39°C cells stopped dividing. This was due to the inactivation of a temperature-sensitive SV40 large T antigen so that it could not bind and repress p53 and retinoblastoma protein. It was established that this proliferation arrest was due to p53. This cell line was then used to identify components of the p53 pathway as cell lines that continue to proliferate after the temperature shift must have reduced expression of p53. Cells cultured at 32°C were infected with the retroviral shRNA library and then after two days cells were shifted to 39°C. Proliferating colonies were picked and the shRNA insert in each was identified by PCR of genomic DNA extracted from cells followed by sequencing. Six genes were identified that prevent p53- dependent growth arrest, including p53, using

this method. In the paper a 'siRNA bar-code screen' is also described, as a quicker method of screening shRNA vector libraries. This method uses the unique 19 nt target sequence that is present in each hairpin as a 'molecular bar code' to measure the relative abundances of shRNAs in two population of shRNA infected cells using microarray analysis.

1.1.3.b. Generation of mouse models

Studies have shown that RNAi technology can be used to generate transgenic mice and that RNAi can be transmitted through the mouse germline. In one example a vector was constructed that expresses hairpin dsRNA targeting the Neil1, which is involved in DNA repair (Carmell *et al.*, 2003). The vector was electroporated into mES cells and absence of the Neil1 protein in stable cells was then confirmed by Western Blotting. Two of these cell lines were injected into blastocysts to produce chimeras. Then these were out-crossed and silencing of Neil1 was confirmed by studying RNA and protein levels. The study concluded that germline transgenic mice can be produced from stable integration of a shRNA expressing vector into mES cells. In another study a lentivirus based vector expressing shRNAs was used to make transgenic and chimeric mice (Rubinson *et al.*, 2003).

In another study a vector expressing shRNA from a H1 polymerase promoter targeting RasGAP was electropored into mES cells (Kunath *et al.*, 2003). The vector also had neomycin resistance and 12 drug resistant ES cell lines were established. Protein levels, analysed by Western blotting, showed a significant reduction in RasGAP in 11 cell lines and only a slight reduction in the other cell line. Embryos were produced from 4 of these ES cell lines, including the line with only a slight reduction in RasGAP, using the tetraploid aggregation method. Embryos were dissected at embryonic day 9.5. It was found that embryos derived from the ES cell line with only a slight decrease in RasGAP expression were the same as the wildtype. Embryos produced from the three ES cell lines that had significantly reduced RasGAP had phenotypes similar to that of the null phenotype.

1.1.3.c. Treatment of diseases and viral infections

In the future it may be possible to use RNAi for treatment of human diseases *in vivo* by knock-down of gene expression of a mutated gene that produces a disease causing

gene product (Pomerantz, 2002). RNAi may also be used for treatment of viral infections. Capodici and colleagues used siRNA duplexes to inhibit HIV-1 infection, and found RNAi can target two points in the viral life cycle (Capodici *et al.*, 2002). These preliminary results show that RNAi has the potential for investigating and discovering new treatments for HIV-1 infection.

The use of RNAi for treatment of the hepatitis B virus was shown in mice (McCaffrey *et al.*, 2003). A vector expressing shRNAs targeting hepatitis B mRNAs was transfected into immunocompetent and immunodeficient mice. A reduction in hepatitis B viral RNA and DNA was seen in the hepatitis B infected mice in the presence of the shRNA.

The potential of using cholesterol conjugated siRNAs for the treatment of coronary artery disease was demonstrated (Soutschek *et al.*, 2004). Apolipoprotein B (ApoB), a protein required for the formation of low density lipoproteins (LDL) in metabolism of cholesterol was targeted. High levels of ApoB, LDL and cholesterol result in an increased risk of coronary artery disease (CAD). Patients with familial hypercholesterolaemia (FH) were shown to have an increase in atherosclerosis and risk of cardiac mortality due to increased levels of LDL and cholesterol. Soutschek *et al.* identified five siRNAs from a screen of 84 that reduced mRNA and protein levels of ApoB by greater than 70%. These chemically synthesised siRNAs contained a partial phosphorothioate backbone and 2'-O-methyl sugar modifications on the sense and antisense strands. The modified siRNAs were shown to be more resistant to exonucleases and endonucleases. Cholesterol was conjugated to the 3' end of the sense strand that was shown to increase the inhibition of the siRNA. There was found to be a reduction in ApoB mRNA levels by 50% in the liver and by 70% in the jejunum (part of the small intestine) when the ApoB siRNAs were injected into mice. This led to a reduction in the levels of cholesterol in the blood and the reduction seen was comparable to levels observed in ApoB knockout mice. Cholesterol containing siRNAs were shown to be more stable than non-conjugated siRNAs and were biologically active *in vivo* unlike unconjugated siRNAs. Therefore the potential of apoB siRNAs for the treatment of CAD was demonstrated.

1.1.3.d. Cancer Therapy

The possibility of using RNAi to make multi-drug resistant cancer cells sensitive to chemotherapy again was investigated (Yague *et al.*, 2004). The *mdr1* gene expresses P-glycoprotein that is an active transporter and acts to efflux drugs from cells. Overexpression of P-protein in cancer cells therefore reduces the toxicity of the cancer drugs such as doxorubicin. In this study a shRNA expressing plasmid targeting *mdr1* was stably integrated into doxorubicin-resistant leukaemia cells. They found that knockdown of MDR1 by RNAi restored the sensitivity of these cells to doxorubicin.

Knockdown of c-Myc in MCF-7 cells (breast cancer cell line) by transfection of myc shRNA expression vectors, decreased the growth rate of cells (Wang *et al.*, 2005). MCF-7 cells that had been transfected with either c-myc shRNA or control vectors were injected into nude mice. The mice were studied for the presence of tumours. A reduction in c-myc by RNAi resulted in a significant reduction in tumours compared to control mice. Therefore c-Myc was highlighted as a therapeutic target for the treatment of breast cancer.

1.2. Murine Embryonic Stem cells

A stem cell is defined as a cell that has the ability for self-renewal in an undifferentiated state and can also differentiate into one or more cell types. There are three types of stem cells that are derived from early mouse embryos; embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981), trophoblast stem cells (Tanaka *et al.*, 1998) and extraembryonic endoderm cells (Kunath *et al.*, 2005).

Mouse ES (mES) cells are obtained from developing explanted blastocysts (Evans and Kaufman, 1981; Martin, 1981). Brook and Gardner (Brook and Gardner, 1997) found the primitive ectoderm or epiblast to be the only source of ES progenitor cells in the late blastocyst. When cultured on feeder layers of mitomycin C treated mouse embryonic or STO fibroblast cells or in the presence of leukaemia inhibitory factor (LIF) (Nichols *et al.*, 1990) mES cells remain pluripotent and retain the ability to proliferate indefinitely without differentiation (Keller, 1995), maintaining a diploid karyotype. This process is called self-renewal and happens via symmetrical cell

division (Chambers and Smith, 2004). When mES cells are cultured in suspension in the absence of feeder layers and LIF they aggregate to form embryoid bodies which subsequently differentiate, forming endoderm, mesoderm and ectoderm. The differentiation of these mES cells has been shown to follow similar patterns of development *in vitro* to development *in vivo* (Desbaillets *et al.*, 2000). Undifferentiated mES cells retain their developmental identity and can be introduced into a host blastocyst and contribute to the germline of the resultant animals resulting in the production of chimeric animals (Bradley *et al.*, 1984).

Therefore mES cells are ideal for studying gene expression in early mouse development. Increased understanding of the pathways involved in the differentiation of ES down different lineages allows for the production of specialised cells from mES cells. These cells can be used for research purposes to understand the role of genes in development and disease. Also dissection of the differentiation pathways may eventually allow production of an unlimited supply of cells for use in medicine.

1.2.1. mES cell self-renewal

Pluripotency of mES cells is maintained by three pathways the effectors being the leukaemia inhibitory factor (LIF), bone morphogenetic proteins (BMPs) and Wnt (Figure 1.4). LIF is a cytokine which acts by binding to the LIF receptor (LIFR), a transmembrane receptor. LIFR dimerises with gp130, a signal transducing receptor. This leads to the phosphorylation of the Janus-associated tyrosine kinases (JAK) and activation of signal transducer and activator of transcription 3 (STAT3) (Humphrey *et al.*, 2004). STAT3 proteins upon phosphorylation form dimers that then translocate to the nucleus leading to the repression of genes that commit cells to mesodermal and endodermal lineages (Niwa *et al.*, 1998; Ying *et al.*, 2003). Myc has recently been shown to be one of the targets of STAT3 involved in maintaining mES pluripotency (Cartwright *et al.*, 2005). Upon differentiation of mES cells Myc mRNA levels were shown to decrease. The activation of STAT3 maintains the pluripotency of mES cells but self-renewal of hES cells is not LIF/STAT3 dependent (Humphrey *et al.*, 2004).

Sato and colleagues suggested that Wnt activation is involved in maintaining the pluripotency of mES cells (Sato *et al.*, 2004). This pathway is followed when Wnt protein binds to the cell surface Frizzled receptor. The downstream signalling results

in inactivation of glycogen synthase kinase 3 (GSK3) leading to the accumulation of nuclear β -catenin that in turn induces transcription of Wnt target genes. The expression levels of *Nanog* are maintained by the Wnt pathway. Aubert and colleagues studied the genes involved in neural differentiation of mES cells (Aubert *et al.*, 2002). They found the Wnt antagonist secreted frizzled-related protein-2 (Sfrp2) to be a positive modulator of neural differentiation. Inhibition of Wnt signalling results in mES cell differentiation down the neural lineage.

BMPs signal via the Smad transcription factors, receptor regulated SMADs 1, 5 and 8 are phosphorylated by the BMP receptor complex and then interact with SMAD4 resulting in translocation of the complex to the nucleus. A major target of the SMADs are the inhibitor of differentiation (Id) genes (Ying *et al.*, 2003). Id expression was shown to prevent differentiation to neural lineages. Smad4 expression promotes mesoderm formation and represses the formation of neuroectoderm (Zhao, 2003). Carpenter and Zernicka-Goetz showed that knockdown of Smad4 in EC cells by RNAi resulted in their differentiation down the neuroectoderm lineage (Carpenter and Zernicka-Goetz, 2004). In order for self-renewal LIF is required together with BMP, when only BMP is present cells differentiate to non-neuronal cell lineages (Ying *et al.*, 2003). When only LIF is present cells differentiate down neuronal cell lineages. BMP 'ES cell self-renewal signalling' is mediated by inhibition of extracellular receptor kinase (ERK) and p38 mitogen-activated protein kinases (MAPK) pathways (Qi *et al.*, 2004).

Paling and colleagues found that LIF induces phosphoinositide 3-kinase (PI3K) and that it is also involved in self-renewal of mES cells (Paling *et al.*, 2004). Inhibition of PI3K led to a decrease in the number of undifferentiated colonies in the presence of LIF as shown by alkaline phosphatase staining. The expression levels of ERKs were found to be increased by inhibition of PI3K whereas there was no observed difference in expression of *Oct-4* or *Stat3*. Inhibition of ERK reverses the effect of PI3K inhibitor on mES cell self-renewal confirming that it also has a role in self-renewal of mES cells.

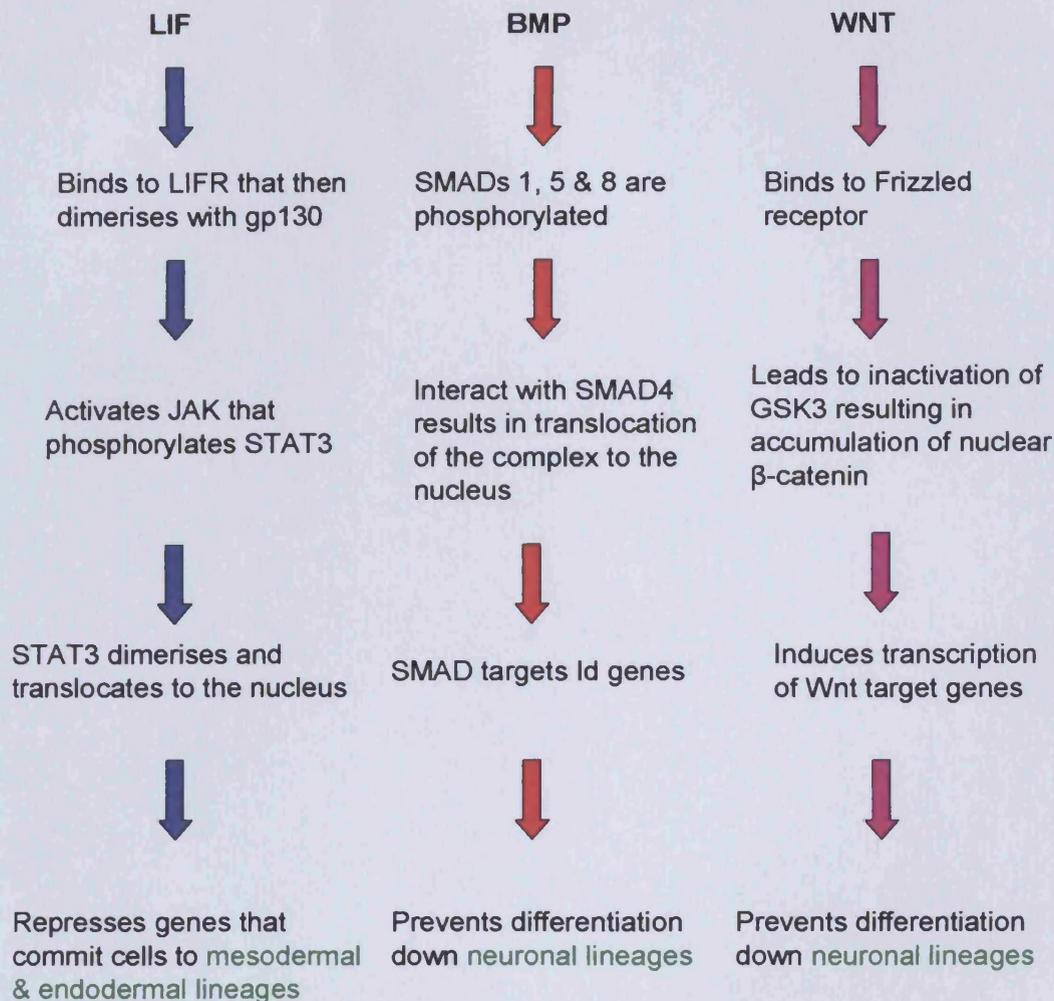


Figure 1.4. Overview of the pathways of the extrinsic regulators of mES cell self renewal. Leukaemia inhibitory factor (LIF) is a cytokine that binds to LIFR that subsequently recruits gp130 to form a trimeric complex. This activates Jak family non-receptor tyrosine kinases that phosphorylate LIFR and gp130. STAT3 then is bound by the complex and is also phosphorylated leading to its dimerisation and translocation to the nucleus. Here it represses genes that commit cells to mesodermal and endodermal lineages. Bone morphogenic protein 4 (BMP4) signal via the SMADs, BMP phosphorylates SMADs 1,5 and 8 that then interact with SMAD4 resulting in translocation of the complex to the nucleus. The Inhibitor of differentiation (Id) genes are targeted by SMADs. Id expression has been shown to prevent differentiation to neuronal lineages. WNT signalling induces transcription of WNT target genes that prevent differentiation down neuronal lineages.

1.2.2. Genes known to be involved in self-renewal of mES cells

1.2.2.a. Oct-4

Oct-4 (also called Pou5f1) is a member of the POU transcription factor family that is expressed in undifferentiated pluripotent cells of the early mouse embryo and in germ cells (Rosner *et al.*, 1990). Oct-4 is encoded by the gene *Pou5f1* and its expression is required for the formation of the inner cell mass (ICM) *in vivo* (Nichols *et al.*, 1998). The levels of Oct-4 in mES cells were shown to be down regulated upon cell differentiation, when LIF is withdrawn from the culture medium (Palmieri *et al.*, 1994).

The level of Oct-4 expression must be maintained within a certain range for mES cell self-renewal (Niwa *et al.*, 1998). An increase in the level of Oct-4 expression (>50%) above normal levels in mES cells was shown to lead to differentiation down endoderm and mesoderm lineages. A decrease in Oct-4 levels (>50% of normal levels) resulted in differentiation of mES cells to trophectoderm. Oct-4 negatively regulates *Cdx-2* and *Hand-1* expressed in trophectoderm cells (Niwa *et al.*, 2000).

1.2.2.b. Nanog

Nanog is another gene involved in maintaining the pluripotency of stem cells independently of the LIF/STAT3 pathway (Mitsui *et al.*, 2003). Over expression of Nanog in mES cells prevents their differentiation in the absence of LIF (Chambers *et al.*, 2003). Nanog prevents differentiation of the mES cells to primitive endoderm and Nanog is thought to repress transcription of genes involved in differentiation such as *Gata4* and *Gata6*. These genes were shown to be upregulated in cells not expressing Nanog. The levels of Nanog are reduced in mES cells upon differentiation.

Lin and colleagues found that p53 binds to the promoter of Nanog and prevents its expression following DNA damage (Lin *et al.*, 2005). Oct-4 expression levels remained constant showing that the reduction in Nanog observed was not due to differentiation of the cells. They proposed that p53 reduces Nanog expression in response to DNA damage leading to differentiation of the cells to allow p53-dependent cell cycle arrest and apoptosis. These processes are inefficient in mES cells after certain types of DNA damage. The regulatory region upstream of the

transcription start site of *Nanog* was found to contain Octamer and Sox elements to which Oct-4 and Sox-2 can bind to respectively.

1.2.2.c. *Sox-2*

Sox-2 has similar patterns of expression as Oct-4 and is expressed in the inner cell mass (ICM), epiblast and germ cells (Avilion *et al.*, 2003). Oct-4 and *Sox-2* act together for transcriptional activation of *Fgf4* in F9 carcinoma cells. Oct-4 and *Sox-2* can act synergistically to control expression of *Fgf4* by binding at adjacent sites within it's the enhancer region (Ambrosetti *et al.*, 2000). *Sox-2* and Oct-4 also act cooperatively together at the promoter regions of *Utf1*, *Sox-2* and *Fbx15* to control their transcription (Ambrosetti *et al.*, 2000; Nichols *et al.*, 1998; Nishimoto *et al.*, 1999; Tokuzawa *et al.*, 2003; Tomioka *et al.*, 2002; Yuan *et al.*, 1995).

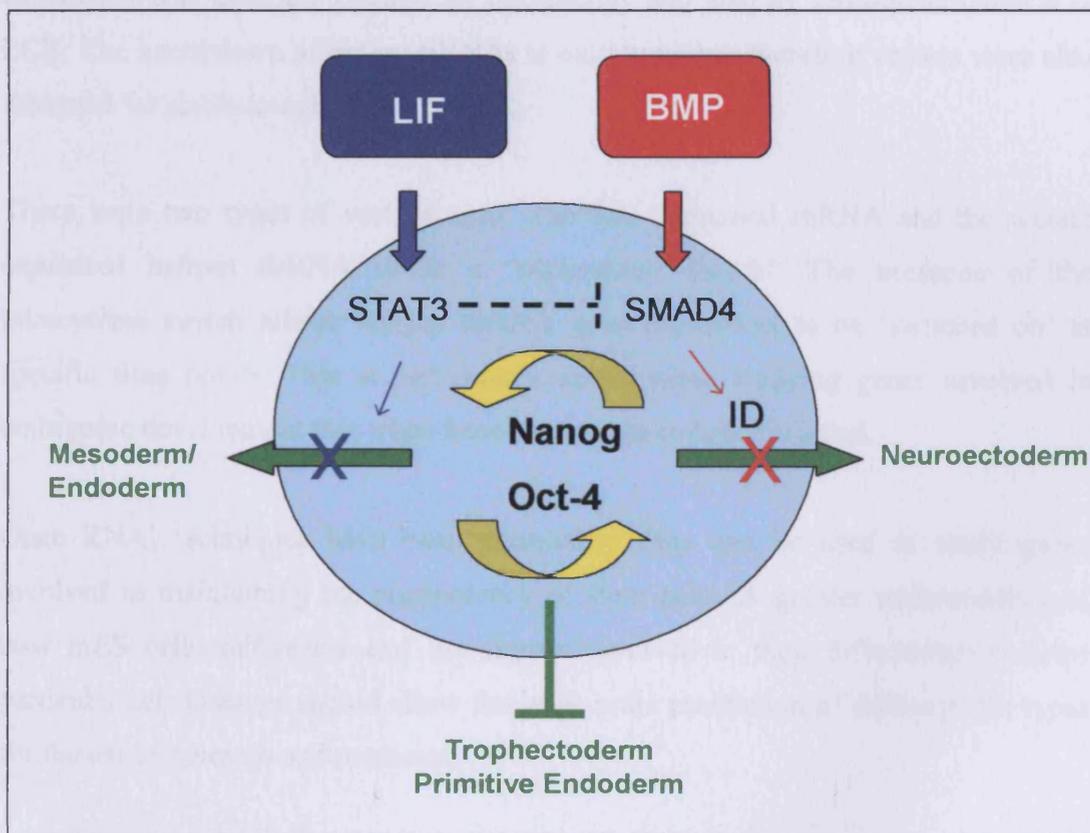


Figure 1.5. Intrinsic and extrinsic factors involved in mES cell self-renewal. LIF activates STAT3 and represses genes that commit cells to non-neural lineages. BMP interacts with SMADs leading to induction of the *Id* genes that prevent differentiation down the neuronal lineage. *Nanog* and Oct-4 are intrinsic transcriptional regulators of ES self-renewal. Reduction in Oct-4 results in the differentiation of cells to trophoctoderm whereas *Nanog* expression prevents differentiation of mES cells to primitive endoderm. [Figure adapted from (Chambers and Smith, 2004)].

1.3. Aims of the Project

RNAi has been shown to be a less time consuming method for studying gene expression in cells and organisms as compared to traditional methods such as antisense technology and production of knockouts by homologous recombination. The aim of the project was to establish RNAi as a tool for studying gene expression in mES cells. Several methods of inducing RNAi in mES cells were investigated including transfection of chemically synthesised siRNAs, the transfection of vectors expressing short hairpin (sh) RNAs and construction of a vector that expressed hairpin dsRNA under tetracycline control.

In preliminary experiments chemically synthesised siRNAs were designed to target endogenously and exogenously expressed genes. Target knockdown of gene expression was assessed visually by microscopy and also by semi-quantitative RT-PCR. The knockdown affect by siRNAs is only transient, therefore vectors were also designed for stable knockdown of genes.

There were two types of vectors used. The first expressed shRNA and the second expressed hairpin dsRNA under a 'tetracycline switch'. The presence of the tetracycline switch allows hairpin dsRNA gene expression to be 'switched on' at specific time points. This is particularly useful when studying genes involved in embryonic development that when knocked out are embryonic lethal.

Once RNAi techniques have been established they can be used to study genes involved in maintaining the pluripotency of stem cells. A greater understanding of how mES cells self-renew and the signals involved in their differentiation down particular cell lineages should allow the large scale production of different cell types for the use in research and medicine.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Materials

For all procedures, solutions were made to the standard required for molecular biology using molecular biology grade and/or “tissue-culture-tested” reagents. All solutions were made using sterile double-distilled water and where appropriate autoclaved or filter sterilised. All tissue culture plates and plasticware was supplied by Nunc, Fisher Scientific, Leicestershire, UK.

The sources of specialised reagents used are listed in Table 2.1. Reagents were of molecular biology grade, unless otherwise stated.

Reagent	Supplier
DNA-Free™ kit, Nuclease free water.	Ambion, Huntingdon, UK.
Sephaglas™ Bandprep Kit.	Amersham Pharmacia Biotech, Buckinghamshire, UK.
Hygromycin B, psiRNA-hH1zeo kit, Zeocin™.	Autogen Bioclear UK Ltd., Wiltshire, UK.
0.4cm gap cuvettes	Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.
pBI-EGFP, pTet-On, 293 Tet-On cell line.	BD Biosciences, Oxford, UK.
Custom synthesised siRNA oligonucleotides, siRNA annealing buffer,	Dharmacon Research Inc., Lafayette, CO.
Ethanol, glycerol, isopropanol,	Fisher Scientific, Leicestershire, UK.
Disposable sterile Universal tubes	Greiner, Stonehouse, UK.
Agarose, DH5α chemically competent <i>E. coli</i> , 1 kilobase plus DNA ladder, Lipofectamine™ 2000 Reagent, OPTI-MEM I reduced serum medium, PBS, 50x TAE, trypsin-EDTA.	Invitrogen Ltd., Paisley, UK.
Ampicillin	Melford Laboratories Ltd, Ipswich, UK.
0.22μM and 0.45μM Nucleopore™ filters	Millipore U.K. Ltd., Watford, Hertfordshire, U.K.
Custom synthesised primers	MWG Biotech, Milton Keynes, UK
Restriction enzymes	New England BioLabs, Hertfordshire, UK.
ABI Prism™ Dye Terminator Cycle Sequencing Reaction Ready Kit Version 3.1.	Perkin Elmer Applied Biosystems, Warrington, UK.

Table 2.1. Biological Reagents

Reagent	Supplier
Agarose, dNTPs (dATP, dCTP, dGTP, dTTP), 100 bp ladder marker, magnesium chloride (25mM), MMLV reverse transcriptase, oligo (dT) ₁₅ primer, <i>Pfu</i> DNA polymerase, pGEM®-T Vector System, Recombinant RNasin Inhibitor, <i>Taq</i> DNA Polymerase, T4 DNA Ligase, Wizard® Plus SV Miniprep DNA purification systems.	Promega Ltd., Southampton, UK.
Plasmid Maxi Kit, QIAquick gel extraction kit, RNeasy® Midi Kit, DyeEX 2.0 Spin Kit.	Qiagen Ltd., West Sussex, UK.
β-mercaptoethanol, BSA, doxycycline, ethidium bromide, LB agar tablets, LB broth, mineral oil, sodium chloride.	Sigma-Aldrich, Dorset, UK.
Gelatin (0.1%).	Stem Cell Technologies Inc., London, UK.

Table 2.1. Biological Reagents (continued)

2.1.1. Autoclaving Conditions

Equipment used when handling DNA or RNA was sterilised by autoclaving 120°C at 15psi for 20 minutes.

2.1.2. Medium preparation

2.1.2.a. For maintaining mammalian cells

Medium	Recipe
ES cell medium	Modified Eagles Medium (MEM) Alpha medium without ribonucleosides and deoxyribonucleosides (Invitrogen Ltd., Paisley, UK). 10% heated inactivated (56°C, 20min) newborn calf serum (NBS) (PAA Laboratories, Yeovil, UK). 10% heat inactivated foetal calf serum (FCS) (PAA Laboratories). 2mM L-glutamine (Invitrogen). 0.1mM β-mercaptoethanol (βME) (Sigma-Alrich, Dorset, UK). Leukaemia Inhibitory Factor (10^3 Uml ⁻¹) (Chemicon, Hampshire, UK).
293 Tet-On cell medium	Modified Eagles Medium (MEM) Alpha medium without ribonucleosides and deoxyribonucleosides (Invitrogen) 10% heat inactivated FCS (PAA Laboratories) 2mM L-glutamine (Invitrogen) 100µg/ml G418 (Invitrogen)
Freezing Mix	10% DMSO (Invitrogen) 90% FCS (PAA Laboratories)

Table 2.2. Cell culture medium composition.

Cell Line	Origin	Supplier
CCB	Mouse ES cell line	Martin Evans, Cardiff University, UK.
ME2	Mouse ES cell line	Martin Evans, Cardiff University, UK.
293 Tet-On™ Cell Line	Human transformed primary kidney (HEK) – derived cell line expressing the reverse tetracycline-controlled transactivator (rtTA).	BD Biosciences, Oxford, UK.

Table 2.3. Cell Lines*2.1.2.b. For bacterial culture*

LB-broth and LB-agar (Table 2.4) were prepared as described by the manufacturer, by addition of the designated pellets to water. Following autoclaving, the medium was left to cool to below 50°C and where required antibiotic was added.

FastMedia™ Zeo Xgal (Autogen Bioclear UK Ltd., Wiltshire, UK) was made up as instructed by the manufacturer. Contents of one pouch were added to 200ml dH₂O and heated in microwave until medium was completely dissolved.

Medium	Recipe
FastMedia™ Zeo Xgal Agar	LB Agar (see below) supplemented with Zeocin™ antibiotic, X-Gal and IPTG.
LB Agar	10g casein enzymatic digest, 5g yeast extract, 5g NaCl, 0.6g Inert binder, 12g agar in 1 litre of ionised water.
LB broth	10g casein enzymatic digest, 5g yeast extract, 5g NaCl, 0.6g Inert binder in 1 litre of ionised water.
SOC medium	20M tryptone, 5M yeast extract, 0.5M NaCl, 1mM MgCl ₂ , 1mM MgSO ₄ , 20mM glucose solution.
TY broth	Bacto-tryptone (20g), bacto-yeast extract (5g), sodium chloride (6g) made up in 1 litre water. pH 7.0 adjusted with NaOH.

Table 2.4. Bacteria culture medium

Bacterial Cell Strain	Supplier
<i>E. coli</i> DH5 α TM	Invitrogen Ltd., Paisley, UK.
<i>E. coli</i> GT116	Autogen Bioclear UK Ltd., Wiltshire, UK.
<i>E. coli</i> GM2163	New England Biolabs, Hertfordshire, UK.

Table 2.5. Bacterial strains

2.1.3. Antibiotics

Stock solution of ampicillin (100mg/ml) made in sterile dH₂O were passed through a 0.22 μ M NucleoporeTM filter (Millipore UK Ltd., Herts, UK) and stored at -20°C.

ZeocinTM and Hygromycin B, were supplied by manufacturer (Autogen Bioclear UK Ltd., Wiltshire, UK) as a 100mg/ml solutions. Puromycin (Autogen Bioclear UK Ltd., Wiltshire, UK) was supplied as a 10mg/ml solution.

2.1.4. DNA Markers

The DNA markers used were 100 bp ladder and 1 kb Plus DNA LadderTM. The fragment sizes of these markers (bp) are given in Table 2.6.

DNA Marker	Fragment Sizes (bp)	Supplier
100bp DNA ladder	1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100	Promega Ltd., Southampton, UK.
1kb Plus DNA Ladder TM	12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1650, 1000, 850, 650, 500, 400, 300, 200, 100	Invitrogen Ltd., Paisley, UK.

Table 2.6 DNA marker fragment sizes (bp) and their respective suppliers.

2.1.5. Oligonucleotide primers

Lyophilised oligonucleotides were resuspended in sterile dH₂O to a workable concentration as specified in the text and stored (-20°C) until required. The sequences of all the primers used are listed in Table 2.7.

In order to minimise mispriming, primers were designed using the OLIGO^{©1991} Primer Analysis Software (Version 4.0, Wojcieck Rychiick, National Biosciences, Plymouth, MN, USA). Primers selected for optimal T_m (melting temperature) at the given primer length were stringently assessed for possible sites of self-complementarity and complementarity to the second primer (especially 3'

complementarity), mainly to reduce the risk of the formation of primer-dimer products.

Name	Sequence (5' to 3')	Annealing temp (°C)
Actb_F	GCTGTATTCCCCTCCATCGTG	60
Actb_R	CTGAGAGGGAAATCGTGCGTG	
Cdx2_F	TTTGTGAGTCCTCCGCAGTA	56
Cdx2_R	GCTGCTGCTTCTTCTTGATT	
EGFP_F	TCGTTGGGGTCTTTGCTCAG	59
EGFP_R	CAAGGACGACGGCAACTACA	
GFP_F (for MmGFP)	AGTGGAGAGGGTGAAGGTGA	59
GFP_R (for MmGFP)	CGTGTCTTGTAGTTCCCGTC	
HActb_F (Raff <i>et al.</i> , 1997)	CCTCGCCTTTGCCGATCC	60
HActb_R	GGATCTTCATGAGGTAGTCAGTC	
Hand1_F	TCATCACCATCATCACCCT	55
Hand1_R	CCATCCGTCTTTTTGAGTTC	
Lamb1_F	CCAGAAAGGAAGACCCGAAGA	60
Lamb1_R	CCATTTTCCGATTGCCACCAG	
LMNA_F	CCGAGTCTGAAGAGGTGGTC	60
LMNA_R	AGGTCACCCTCCTTCTTGGT	
LMNA hp_F	TGAGATGCTGCGGCGGGT	58
LMNA hp_R	AGTATGAGATGCTGCGGC	
LMNA IR_F	GGCCACTAGTATGAGATGCTGCGGC	68
LMNA IR_R	GGCCTTCGAACTCACGCTGCTTCCC	

Table 2.7. Sequences of primers used in PCR reactions or for sequencing (All primers were purchased from MWG Biotech, Milton Keynes, UK).

Name	Sequence (5' to 3')	Annealing temp (°C)
Linker_F	GGACCATTGCACTCGAGC	58
Linker_R	GCTCGAGTGCAATGGTCC	
MmGFP_F	AGACACGTGCTGAAGTCAAG	63
MmGFP_R	CCATGCCATGTGTAATCCCA	
Oct-4_F	TGCCGTGAAGTTGGAGAAGGT	60
Oct-4_R	GCTGATTGGCGATGTGAGTGA	
Oct-4 IR_F	GGCCACTAGTATGGAACAGTTTGCC	64
Oct-4 IR_R	GGCCTTCGAACTTCGGGCACTT	
OL381	CCCTAACTGACACACATTCC	57
pBI-MCS_F	ACGCTGTTTTGACCTCCATAG	56
pBI-MCS_R	AGAAAGAACAATCAAGGGTCC	
psiRNA_F	CACTGGTAACAGGATTAGCAG	58
psiRNA_R	AGGCCCTGCAGGTTAATTAAG	
Rex-1_F	GCCAGTCCAGAATACCAGAGT	60
Rex-1_R	CTTTGCGTGGGTTAGGATGTG	
rtTA_F	ACCATACTCACTTTTGCCCTT	56
rtTA_R	GTAAACATCTGCTCAAACCTCG	

Table 2.7. Sequences of primers used in PCR reactions or for sequencing (continued) (All primers were purchased from MWG Biotech, Milton Keynes, UK).

2.1.6. Reagents and Buffers

All routine laboratory solutions were prepared using dH₂O. Sterilisation was achieved by autoclaving 120°C at 15psi for 20 minutes where required. Heat sensitive components were passed through 0.22µm Nucleopore™ filters (Millipore UK Ltd., Herts, UK) and added separately following autoclaving.

Procedure	Solution	Components
Agarose gel electrophoresis (Section 2.2.1)	Electrophoresis loading dye	0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400; Pharmacia) in water
	TAE (1x)	Tris-acetate (40mM), EDTA (1mM)
RNA preparation using Qiagen RNeasy midi kit (Section 2.2.2)	Buffer RLN	50mM Tris.Cl, pH 8.0 140mM NaCl 1.5mM MgCl ₂ 0.5% (v/v) Nonidet® P-40 (1.06g/ml)
	Buffer RLT	Protected recipe
	Buffer RW1	Protected recipe
	Buffer RPE	Protected recipe
Mini Preparation of DNA using Promega Wizard® SV Miniprep DNA Purification System (Promega Ltd., Southampton, UK) (Section 2.2.6a)	Cell Resuspension Solution	50mM Tris-HCl (pH 7.5) 10mM EDTA 100µg/ml RNase A
	Cell Lysis Solution	0.2M NaOH 1% SDS
	Neutralisation Solution	4.09M guanidine hydrochloride 0.759M potassium acetate 2.12M glacial acetic acid Final pH is approximately 4.2.
	Column Wash Solution	60mM potassium acetate 8.3mM Tris-HCl (pH 7.5) 0.04mM EDTA (pH 8.0) 60% ethanol
Maxi Preparation of DNA Using Qiagen Maxi Kit (Section 2.2.6b)	Buffer P1	50mM Tris.Cl, pH 8.0 10mM EDTA, 100µg/ml RNase A
	Buffer P2	200mM NaOH, 1% SDS
	Buffer P3	3.0M potassium acetate, pH 5.5
	Buffer QC	1.0M NaCl, 50mM MOPS, pH 7.0, 15% Isopropanol
	Buffer QBT	750mM NaCl, 50mM MOPS pH 7.0, 15% Isopropanol, 0.15% Triton® X-100
	Buffer QF	1.25M NaCl 50mM Tris.Cl, pH 8.5 15% Isopropanol

Table 2.8. Solutions and reagents

Procedure	Solution	Components
Extraction of DNA from agarose gels using the Sephaglas™ Bandprep Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) (<i>Section 2.2.11</i>)	Sephaglas BP	20% (w/v) Sephaglas BP suspended in distilled water containing 0.15% Kathon® CG/ICP Biocide.
	Gel Solubiliser	Buffered solution containing NaI.
	Wash Buffer	20mM Tris-HCl (pH 8.0), 1mM EDTA and 0.1mM NaCl solution to which 18ml of absolute ethanol must be added before use.
	Elution Buffer	10mM Tris-HCl (pH 8.0), 1mM EDTA.
Ligation of inserts to digested plasmid DNA (<i>Section 2.2.13</i>)	T4 DNA ligase buffer	300mM Tris-HCl (pH 7.8) 100mM MgCl ₂ 100mM DTT 10mM ATP
Preparation of Chemically Competent Cells (<i>Section 2.2.14</i>)	TFB1 Solution	Potassium acetate (30mM), magnesium chloride (50mM), potassium chloride (100mM), calcium chloride (10mM). pH 5.8.
	TFB2 Solution	Sodium-MOPS pH 7.0 (10mM), calcium chloride (75mM), potassium chloride (10mM), 20% glycerol.

Table 2.8. Solutions and reagents (continued)

2.2 Tissue Culture

2.2.1 Maintenance of cells

Mouse ES (mES) cells were cultured on tissue-culture grade plastic-ware pre-coated with 0.1% gelatin (StemCell Technologies Inc., London, UK) in mES cell medium (Table 2.2) in a humidified incubator at 37°C with 5% CO₂. For optimum growth of mES cells, plates of cells were not seeded too sparsely or allowed to become too confluent. Less favourable conditions result in the selection of abnormal cells, containing chromosome rearrangements.

293 Tet-On cells were also cultured on tissue-culture grade plastic-ware pre-coated with 0.1% gelatin (StemCell Technologies Inc. London, UK) in medium (Table 2.2).

2.2.1.a. Thawing cells

A vial of cells taken from liquid nitrogen was thawed quickly by incubating in a 37°C waterbath. The cells were transferred to a 15ml Falcon tube and medium (10ml) was added to the cells. Cell suspension was transferred to a 100mm dish pre-coated with 0.1% gelatin (StemCell Technologies Inc., London, UK). Medium was changed on the cells the next day and cells were ready for splitting approximately 48h after thawing.

2.2.1.b. Passaging of cells

ES cells were routinely split between 1:3 and 1:6 every 2-3 days when cells reached 70% confluency to maintain exponential growth. Medium was aspirated, cells were washed with PBS (Invitrogen Ltd., Paisley, UK) and then trypsin-EDTA (Invitrogen Ltd., Paisley, UK) (1ml for a 100mm dish) was added. When cells were rounded and detached from the bottom of the dish, trypsin was inactivated by the addition of growth medium (9ml). Cells were pelleted (1500 x g for 5min) and the supernatant was removed. Pellet was resuspended in medium (10ml). Cells were counted using a haemocytometer and calculating the number of cells per ml. The appropriate numbers of cells were seeded into pre-gelatinised plates for passage/transfection.

2.2.1.c. Freezing down cells for storage in liquid nitrogen

Cells were removed from the bottom of the dish as described in Section 2.1.1.b. Pelleted cells were resuspended in freezing mix (Table 2.2) with between 5×10^6 and 1×10^7 cells per ml. Cell suspension was transferred 1ml per cryovial. Cells were frozen slowly by placing them in an insulated box at -80°C for at least 2 days before transferring them to liquid nitrogen.

2.2.2. Transfection methods2.2.2.a. Lipofectamine™ 2000 transfection

This transfection method was used for transient transfection of siRNAs and plasmid DNA into cells that had been seeded into either 24-well (6×10^4 cells per well) or 6-well plates (2×10^5 cells per well) 24h prior to transfection.

On the day of transfection the following were mixed in 1.5ml microcentrifuge tubes (see Table 2.9 for volumes):

- 1) Plasmid DNA/siRNA duplex and OPTI-MEM I reduced serum medium (Invitrogen Ltd., Paisley, UK).
- 2) Lipofectamine™ 2000 Reagent (Invitrogen) and OPTI-MEM I reduced serum medium (Invitrogen).

Control transfections were also set up containing the transfection mix only.

Culture plate	Volume of plating medium (ml)	DNA/siRNA (μg) in OPTI-MEM® vol (μl)	Lipofectamine™ 2000 (μl) in OPTI-MEM® vol (μl)
24-well	0.5	up to $0.8\mu\text{g}$ in $50\mu\text{l}$	$2.0\mu\text{l}$ in $50\mu\text{l}$
6-well	2	up to $4.0\mu\text{g}$ in $250\mu\text{l}$	$4.0\mu\text{l}$ in $250\mu\text{l}$

Table 2.9. Volume of reagents used when performing Lipofectamine™ 2000 transfections

After incubation for 5min at room temperature the two solutions were combined, mixed gently and incubated for 20min (room temperature) to allow DNA-Liposome complexes to form. The complexes were then added to each well of cells. Transfection mix was removed from the cells and replaced with fresh medium 24h later.

2.2.2.b. Calcium phosphate transfection

This method was used for the cotransfection of CCB cells with psiOct-4/psi and pTP6 (Chapter 4) and also for the generation of the cell lines METP6 (Chapter 3) and 293 Tet-On LMNAIR (Chapter 5).

The following reagents were used:

- 2X HEPES-buffered saline (HBS), (280mM NaCl, 10mM KCl, 1.5mM Na₂HPO₄ · 2H₂O, 12mM dextrose, 50mM HEPES). The pH was adjusted to 7.05 with 0.5 N NaOH and then the volume was made up to 100ml with distilled H₂O. The HBS solution was sterilised by passing it through a 0.22µm Nucleopore™ filter (Millipore UK Ltd., Herts, UK). It was stored in 5ml aliquots at -20°C until required.
- 2M CaCl₂ The solution was sterilised by passing it through a 0.22µm Nucleopore™ filter (Millipore UK Ltd., Herts, UK).

Cells were seeded with 5×10^5 cells in 60mm plates in normal growth medium 24h before transfection. For each transfection up to 6µg DNA was made to 219µl with ddH₂O and mixed with 31µl 2M calcium chloride in a 15ml Falcon tube (Fisher Scientific, Leicestershire, UK) HBS (2x) (250µl) was added to the calcium chloride mixture slowly dropwise whilst bubbling air through the mixture with a 5ml pipette. The transfection mix was incubated at room temperature for 20-30 min. During this time cells were washed once with serum free medium and 4.5ml OPTI-MEM I reduced serum medium (Invitrogen Ltd., Paisley, UK) was added to each dish. The calcium phosphate-DNA suspension (500µl) was added dropwise to the cells. Cells were incubated with transfection mix for 5h at 37°C in 5% CO₂ incubator. Transfection mix was then removed and replaced with 5ml normal cell medium.

2.2.2.c Electroporation of cells

To generate cell lines that were stably transfected with the plasmids psiRNA_Rex-1, psiRNA_Lamb1, and pTet-On, CCB cells were electroporated with linearised plasmid DNA. Plasmid DNA was prepared by linearising with either *Cla*I (psiRNA_Rex-1, psiRNA_Lamb1) or *Sca*I (pTet-On) (Section 2.3.10.b). Plasmid DNA was gel purified (Section 2.2.11) and ethanol precipitated (Section 2.2.12).

Medium was changed on the ES cells 3h prior to electroporation. Cells were harvested by trypsinisation and washed once with PBS. They were then counted and 10^7 cells were pelleted and resuspended in PBS (500 μ l). Linearised construct (20 μ g) was added to the suspension and incubated on ice (5min). Suspension was transferred to a 0.4cm gap cuvette (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and electroporated at 200V, capacitance 960 μ F in a Bio-Rad Gene Pulser™ electroporator. Cuvette was then incubated on ice (20min). Cell suspension was added to 9.5ml normal ES cells medium and used to seed ten 100mm gelatin-coated plates.

2.2.3. Determining the optimum concentration of selection reagent

Cells (2×10^5) cells were seeded into 100mm dishes containing 10ml of the appropriate culture medium plus varying amounts (0, 50, 100, 200, 400, 800 μ g/ml) of Hygromycin B, Zeocin™ (Autogen Bioclear UK Ltd., Wiltshire, UK) and G418 (Invitrogen Ltd., Paisley, UK). Cells were incubated (37°C, 5% CO₂) for 10-14 days, replacing the selective medium every 4 days. Dishes were examined for viable cells every two days. For selecting stable transformants, the lowest concentration that begins to give massive cell death in ~5 days and kills all the cells within 2 weeks was used.

Antibiotic	Concentration used in selection medium (μ g/ml)
Neomycin	200
Zeocin™	5
Puromycin	3
Hygromycin B	200

Table 2.10 Concentrations of antibiotics used for selection of stably transformed cell lines.

2.2.4. Picking ES cell colonies of stably transformed cells

Post-transfection (48h), fresh medium containing antibiotic at appropriate concentration (Table 2.10) was added to transfected cells. Antibiotic containing medium was replaced every 3-4 days. The formation of cell colonies was seen after 7 days of selection and individual colonies were picked after 14 days as surviving colonies should contain a functional antibiotic resistance gene. All cells on control plates (cells not transfected) had died by this time.

A flat bottomed 96-well plates was coated with gelatin then growth medium containing the selection reagent was added to each well (100 μ l). A sterile round bottomed 96-well plate with aliquots of 20 μ l trypsin-EDTA (Invitrogen Ltd., Paisley, UK) was also prepared.

Medium was aspirated from the plates containing the colonies. Cells were washed with PBS (Invitrogen Ltd., Paisley, UK) and then covered with 10ml of PBS. Using a dissecting microscope colonies were picked using a 200 μ l pipette set at 5 μ l and each were transferred to a well in the round-bottomed 96-well plate containing trypsin-EDTA. Cells were resuspended by pipetting up and down to break up the colony into single cells then 80 μ l growth medium was added to each well using an 8-way pipette. Cell suspension (100 μ l) was transferred into a well of the flat bottomed multi-well plate. Cells were incubated in 37°C, 5% CO₂ incubator. Medium was changed on the cells the next day and when a healthy culture was established (3-4 days). Cells were passaged to expand cells for RNA/DNA extraction and for freezing down.

2.2.5. Culturing resistant cell clones

When the cells had become confluent in the 96-well plate they were moved individually to 6-well plates, using antibiotic selection containing medium. All of the clones grew at different rates and therefore were examined individually on a daily basis. A vial of each cell line was frozen down for future use and cells were harvested and DNA/RNA extracted from them for analysis.

2.3. Molecular Biology

2.3.1. Agarose Gels

DNA/RNA samples were resolved by gel electrophoresis. Agarose gels were prepared by melting the required amount of agarose (1-2% w/v) (Electrophoresis Grade, Invitrogen Ltd., Paisley, UK) dissolved in 1 x TAE. When the gel mix was below 50°C, ethidium bromide was added to a final concentration of 0.5 μ g/ml. The appropriate DNA/RNA was mixed with loading dye and loaded onto the gel. For DNA gels the appropriate molecular weight marker was also loaded (Table 2.6) to ascertain size of PCR products. Electrophoresis was performed at the required voltage

in a gel tank in 1 x TAE buffer. Nucleic bands were then visualised under UV light and a photograph was taken.

2.3.2. Preparation of cytoplasmic RNA

Cytoplasmic RNA was isolated from cells using either the RNeasy[®] Mini Kit (Qiagen Ltd., West Sussex, UK). Cells (between 4×10^5 and 1×10^7 that had been trypsinised and resuspended in medium (Section 2.2.1.b) were pelleted ($1500 \times g$, 5min) and supernatant was discarded. The pelleted cells were resuspended in cold (4°C) Buffer RLN ($175\mu\text{l}$) that lyses the cell membrane and incubated on ice for 5min. The lysate was centrifuged at 4°C ($300 \times g$, 2min). The supernatant containing the cytoplasmic extract was transferred to a new microcentrifuge tube. Buffer RLT ($600\mu\text{l}$) was added to the sample and mixed thoroughly by vortexing. Ethanol ($430\mu\text{l}$) was then added and mixed by pipetting and the sample was applied to an RNeasy mini column placed in a 2ml collection tube. This was centrifuged at $8000 \times g$ for 15secs and the flow through was discarded. Buffer RW1 ($700\mu\text{l}$) was added and the tube was centrifuged ($8000 \times g$, 15secs) to wash the column. RNeasy column was transferred to a new 2ml collection tube and Buffer RPE ($500\mu\text{l}$) was added and centrifuged ($8000 \times g$, 15secs), flow-through was discarded. This wash step was repeated. The column was transferred to a new sterile 1.5ml microcentrifuge tube and RNA was eluted from the column by the addition of RNase-free water ($50\mu\text{l}$) directly to the spin-column membrane. Then after standing for 1min the tube was centrifuged ($8000 \times g$, 1min). RNA was stored at -70°C until required.

2.3.3. DNase treatment of RNA

Contaminating DNA was removed from RNA preparations before reverse-transcription and PCR analysis was performed using the DNA-free[™] kit (Ambion, Huntington, UK). RNA ($30\mu\text{l}$) that had been extracted from cells (Section 2.3.2) was combined with 10 x DNase I Buffer ($3\mu\text{l}$) and DNase I ($1\mu\text{l}$). Solution was mixed gently and incubated at 37°C for 30min. DNase inactivation reagent was resuspended by vortexing the tube then added ($5\mu\text{l}$) to each DNase treated RNA sample. The tubes were incubated at room temperature for 2min during this time tube was flicked once to re-disperse the DNase Inactivation Reagent. The tube was then centrifuged ($10,000 \times g$, 1min) and RNA solution was transferred to a clean microcentrifuge tube.

2.3.4. Reverse Transcription

DNase-treated RNA (1µg) was added to sterile H₂O to a total volume of 26.5µl. Sample was heated to 70°C for 5 min to denature the RNA. Reverse transcription reaction components were then added [1mM dNTPs, 125ng Oligo (dT)₁₅ primer, 1 x MMLV Reaction Buffer, 100mM Dithiothreitol (2.5µl), 20U of Recombinant RNasin Ribonuclease Inhibitor, 400U MMLV Reverse Transcriptase (Promega Ltd., Southampton, UK) made up to 23.5µl with sterile water] as a cocktail. Reactions were incubated at 37°C for 1.5h. Heating to 95°C for 5 min terminated the reaction.

Synthesised cDNA was stored -20°C prior to downstream applications such as PCR amplification.

2.3.5. PCR Amplification

PCR reaction were performed using a reaction mixture containing the target DNA, 1x *Taq* buffer, 2.5mM magnesium chloride, 0.2mM dNTPs, 0.4µM of each primer (Table 2.7), 0.05units/µl *Taq* polymerase and nuclease-free water to make up the final volume. Preparative mixtures were contained in 500µl thin walled PCR tubes and overlaid with mineral oil. Denaturing, annealing and chain extensions were performed in a Biometra® UNO-Thermoblock™ (Biometra, Thistle Scientific, Glasgow, UK) programmed according to the required conditions. Following amplification, the aqueous layer was transferred to a fresh tube for analysis.

In PCR amplification of DNA for cloning *Pfu* DNA polymerase (0.03U/µl) was also added to the reaction mix. This enzyme was included as it exhibits 3' to 5' exonuclease (proofreading) activity. It was used for polymerase reactions requiring high fidelity synthesis such as in cloning.

In semi-quantitative PCRs amplification was limited to the least number of cycles for visualisation using the Gel Doc (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). This was to measure varying levels of expression between different samples when cDNA had been normalised against the housekeeping gene, beta actin (*Actb*). PCR reactions were performed in parallel at least twice to ensure consistent and reproducible results.

2.3.6. Plasmid DNA preparation

2.3.6.a. Small scale preparation of plasmid DNA

Plasmid DNA was prepared using the Promega Wizard® PlusSV Minipreps Kit (Promega Ltd., Southampton, UK) according to the manufacturer's instructions. A single bacterial colony was inoculated into 5ml of LB medium containing the appropriate antibiotic selection. The culture was incubated with constant agitation (37°C overnight at 250rpm). A proportion of the cells (1.5ml) were pelleted by centrifugation (10000 x g for 10min) and the supernatant was removed. The cells were resuspended in 250µl resuspension solution (50mM Tris-HCl (pH 7.5), 10mM EDTA, 100µg ml⁻¹ RNase A) and transferred to a sterile microcentrifuge tube.

This suspension was lysed by the addition of 250µl lysis solution (200mM NaOH, 1% (w/v) SDS) and all endonucleases removed by incubating the cells in the presence of 10µl alkaline protease solution (5min). Neutralising solution (350µl) (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid) was subsequently added and all cell debris was pelleted by centrifugation (10000 x g, 10min). The supernatant containing the plasmid DNA was placed in a Wizard® plus SV spin column and centrifuged (10000 x g, 1min). The resin bound plasmid DNA was washed twice with 1ml of column wash solution (60% (v/v) ethanol, 10mM Tris-HCl (pH 7.5), 60mM potassium acetate) by centrifugation (10000 x g, 1min). Sterile dH₂O (30µl) was then added and the plasmid DNA eluted into a clean microfuge tube by centrifugation (10000 x g, 1min). The DNA was stored at -20°C until required.

2.3.6.b. Large scale preparation of plasmid DNA

DNA was isolated using Qiagen Plasmid Maxi Prep Kit as described in the manufacturers protocol (Qiagen Ltd., West Sussex, UK). A single colony was picked from a freshly streaked selective LB-Agar plate and inoculated in a starter culture of 5ml LB medium containing the appropriate selective antibiotic. Culture was incubated for 8h at 37°C with shaking at 250rpm. The starter culture was diluted 1/500 into the selective LB medium then incubated at 37°C for 12-16h with shaking at 250rpm. Bacterial cells were harvested by centrifugation (6000 x g for 15min at 4°C). The bacterial pellet was resuspended in Buffer P1 (10ml) and then Buffer P2 (10ml) was added. The suspension was mixed gently but thoroughly by inverting the tube 4-6

times and then was incubated at room temperature for 5min. Chilled Buffer P3 (10ml) was added and mixed immediately but gently by inverting 4-6 times followed by incubation on ice for 20min. The solution was then centrifuged (20000 x g, 30min at 4°C). The supernatant containing the plasmid DNA was removed promptly. The supernatant was centrifuged again (20000 x g, 15min at 4°C). The supernatant containing plasmid DNA was removed promptly. A QIAGEN-tip 500 was equilibrated by applying Buffer QBT (10ml) and the column was allowed to empty by gravity flow. The supernatant containing the plasmid DNA was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with 2 x 30ml Buffer QC. DNA was eluted with Buffer QF (15ml). DNA was precipitated by the addition room temperature isopropanol (10.5ml) to the eluted DNA. Solution was mixed and centrifuged immediately (15000 x g, 30min at 4°C). The supernatant was carefully decanted and discarded. DNA pellet was washed with 70% ethanol (5ml) and centrifuged (15000 x g, 10min). The supernatant was decanted without disturbing the pellet. The pellet was air-dried for 10min and the DNA was dissolved in a suitable volume of buffer TE, pH 8.0. Purified plasmid was stored at -20°C until required.

2.3.7. Genomic DNA preparation from cells

A 100mm plate of cells was trypsinised to remove cells from the bottom of the plate as previously described (Section 2.2.1.b). Cell suspension was centrifuged (10000 x g, 5 min). The pellet was resuspended in PBS (1ml), transferred to a microcentrifuge tube and centrifuged (10000 x g, 10min). PBS was aspirated leaving a little behind to resuspend the pellet by flicking the tube. Proteinase K lysis buffer (200µl) and proteinase K to a final concentration of 250µg/ml was added to the resuspended pellet. The tube was inverted gently and incubated at 55°C for at least 3-4h, with regular inversion. After this time 70% acetone / 5% DMF at -20°C (1ml) was added with a glass pipette. The tube was inverted vigorously to get DNA out of solution then centrifuged (10000 x g, 10min). The pellet was washed twice with 70% ethanol and then dried at room temperature. The precipitated DNA was dissolved in TE pH8.0 (200µl) by heating to 70°C. DNA was stored at -20°C until needed.

2.3.8. Quantification of nucleic acid concentrations

Nucleic acids absorb at a wavelength of 260nm. Given that an optical density (OD) of 1 unit is equivalent to 50 $\mu\text{g ml}^{-1}$ of double-stranded DNA or 40 $\mu\text{g ml}^{-1}$ single-stranded DNA and RNA, it was possible to quantify DNA and RNA concentrations. Furthermore, determining absorbencies at 260nm and 280nm provides a means of examining sample purity. Pure preparations of DNA and RNA will have an A_{260}/A_{280} ratio of approximately 1.8 and 2.0 respectively. Any protein or phenol contaminants will lower these values. Likewise a A_{260}/A_{280} ratio of less than 2 is considered to be indicative of ethanol or salt contamination (Sambrook *et al.*, 1989). Optical density readings were performed on a UV-visible spectrophotometer [CamSpec M330 (CamSpec, Cambridge, UK)] using a quartz cuvette.

2.3.9. Annealing of oligonucleotides

Sense (1.7 μM) and antisense (1.7 μM) oligonucleotides were combined together with sodium hydroxide (0.1M) made up to a total volume of 30 μl with sterile water. They were then heated in a water bath (80°C, 10 min). Primers were allowed to cool slowly by leaving them in the water bath after it had been switched off.

Oligonucleotide	Sequence (5'-3')
pBI MCS S	CGCGTCGGGACTAGTCCCGATCGATGGACCATTGCACTCGA GCGGACTTCGAAGCAT
pBI MCS AS	CGCGCATGCTTCGAAGTCCGCTCGAGTGCAATGGTCCATCG ATCGGGACTAGTCCCG

Table 2.11 Sequences of oligonucleotides that were annealed together and subsequently cloned into pBI-EGFP vector (Clontech, Oxford, UK). (Oligos were purchased from MWG, Biotech, Milton Keynes, UK).

2.3.10. Restriction Digests

2.3.10.a. Analytical

Analytical restriction digests were performed to confirm successful ligation of the relevant insert using enzymes purchased from New England BioLabs (Hertfordshire, UK). For analytical digests 200-500ng of plasmid DNA was set up in 50 μl digest. The following components mixed together X μl DNA, 5 μl of appropriate enzyme buffer, bovine serum albumin (100 $\mu\text{g/ml}$, if required), 1 μl restriction enzyme, sterile H₂O to

a final volume of 50 μ l. Digests were incubate at the optimum temperature for about 2-3h.

2.3.10.b. Preparative Restriction Digest

Restriction digest were formed on a larger scale when preparing linearised plasmid DNA for transfection or for cloning. Restriction enzyme nuclease digests of 1-10 μ g plasmid DNA, 4 μ l restriction enzyme, 5 μ l enzyme buffer and bovine serum albumin (100 μ g/ml, if required) were made up to 50 μ l with sterile water in microcentrifuge tubes. Digestions requiring two restriction endonucleases contained 2 μ l of each enzyme and 1 x enzyme buffer compatible for the two enzymes made up to 50 μ l with sterile water in microcentrifuge tubes. Reactions were incubated 4-6h at the appropriate temperature.

2.3.11. Extraction of DNA from agarose gels

DNA was run on an agarose gel was made and run as described previously (Section 2.3.1). The band containing the DNA of interest (visualised using UV light) was excised using a scalpel and transferred to a preweighed 1.5ml microcentrifuge tube. The mass of the gel slice was determined and DNA was extracted from agarose gel slice using either the Sephaglas™ Bandprep Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) for DNA fragments >200bp or the QIAquick gel extraction kit (Qiagen, West Sussex, UK) for fragments 70-200bp. Protocol were as provided by the manufacturers.

When using the Sephaglas™ Bandprep Kit with gel slices <250mg 250 μ l gel solubiliser was added, the sample vortexed and incubated at 60°C until the agarose slice was dissolved. The container of Sephaglas BP was vortexed vigorously to form a uniform suspension Then Sephaglas BP (5 μ l) was added to the dissolved gel slice and vortexed gently. The tube was pulsed for 30sec in a microcentrifuge and then supernatant was removed and discarded. The pulse spin was repeated and any residual liquid was removed, taking care not to disturb the pellet. The pellet was resuspended in wash buffer (80 μ l) by pipetting up and down several times. The sample was pulsed for 30secs and the supernatant was removed. This wash step was repeated twice more. The tube was tapped to disperse the pellet then left to air-dry for at least 10min.

Elution buffer (20 μ l) was added to the dried pellet and the microcentrifuge tube was vortexed gently to resuspend the pellet. After incubation at room temperature (5min) with periodic agitation the sample was centrifuged (1000rpm, 1min). The supernatant containing the purified DNA was transferred to a sterile microcentrifuge tube, taking care not to disturb the pellet. The eluted DNA was stored at -20°C until required. For gel slices >250mg the reagents added were scaled up as described in the provided protocol.

When using the QIAquick gel extraction kit 3 volumes of Buffer QG was added to 1 volume of gel (100mg~100 μ l) and then incubated at 50°C until the gel slice had dissolved. 1 gel volume of isopropanol was added to the sample and mixed and then the solution was applied to a QIAquick column that had been placed in a 2ml collection tube. The column was centrifuged (1 min), the flow-through was discarded and then Buffer QG (0.5ml) was added to the column and centrifuged again (1min). The column was washed by the addition of Buffer PE (0.75ml) to the column and centrifuging for 1 min. After the flow through had been discarded the column was centrifuged (17,900 \times g, 1min) to remove any residual ethanol. The column was placed into a clean 1.5ml microcentrifuge tube and DNA was eluted by the addition of 50 μ l of sterile H₂O and the column was centrifuged for 1min.

2.3.12. Purification of DNA

DNA that was to be transfected into cells was cleaned up by ethanol precipitation. DNA was precipitated by adding 2 volumes of 100% ethanol and 0.1 volumes of 3M sodium acetate (pH4.8) to the DNA solution and incubating at -80°C for 30min. The DNA was recovered by centrifugation (13000 \times g for 10min). The supernatant was removed and the pellet was washed with 70% ethanol then the DNA was pelleted again (13000 \times g for 10min) and the supernatant was discarded. The pellet was air dried then resuspended in sterile H₂O.

2.3.13. Ligation of insert DNA into digested plasmid

PCR products and oligonucleotides were annealed to digested plasmid using T4 DNA ligase (Promega Ltd., Southampton, UK). Ligation mix was made up of fragments to be annealed, T4 DNA ligase buffer (1 μ l), T4 DNA ligase (1 μ l), H₂O (to make a final

volume 10 μ l). A molar ratio of 3:1 of insert to plasmid was used in the ligations calculated as follows:

$$\text{ng of insert} = \frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \frac{3}{1}$$

Ligation reactions were incubated at 4°C for 16h.

2.3.14. Preparation of chemically competent *E. coli* cells

A single colony was used to inoculate a 5ml TY broth (Table 2.4) and incubated at 37°C in an shaking incubator (250rpm, 2h). Culture was added to TY broth (100ml) and incubated again (37°C, 250rpm) until absorbance at 600nm was equal to 0.5. Culture was centrifuged (2500rpm, 5min) and supernatant was removed. Cell pellet was resuspended in cold TFB1 solution (25ml) (Table 2.8) incubated on ice for 1h. Cell suspension was centrifuged again (2000rpm, 5min at 4°C). Cell pellet was gently resuspended in cold TFB2 solution (4ml) (Table 2.8) and incubated on ice for 1h. Cells were then aliquoted into sterile microtubes and stored at -70°C until needed.

2.3.15. Transformation of chemically competent *E. coli* bacteria

Newly ligated plasmids were transformed into chemically competent *E. coli* (Table 2.5). Aliquots of competent cells were thawed on ice. The ligated sample (3 μ l) was added to the thawed 50 μ l competent cells and mixed then incubated on ice for 30min. Samples were heat-shocked at 37°C for 20secs and then placed on ice for a further 2min. SOC medium (950 μ l) (Table 2.4) was added at room temperature and bacteria were incubated in an orbital incubator (37°C at 250rpm) for 1h.. Transformation (150 μ l) was spread on an LB agar plate containing the appropriate antibiotic selection. Plates were inverted and incubated at 37°C overnight and colonies picked the next day.

2.3.16. PCR Screening of bacterial colonies

PCR screening is used to determine whether or not ligation into a vector has been successful by PCR amplification of the relevant insert gene. Bacterial colonies were picked from plates spread 24h previously with transformed bacteria and used to inoculate 1.5ml broths containing the appropriate antibiotic selection agent. These were incubated with constant agitation (37°C for 5h at 250rpm). Culture (0.5ml) was

pelleted (12000 x g, 10min) in a sterile microcentrifuge tube. The supernatant was removed and pellet resuspended in 50µl of sterile water. Cells were lysed (95°C for 10 min), then debris was pelleted (12000 x g, 5min at room temperature). Supernatant containing the plasmid DNA was transferred to another microcentrifuge tube. PCR analysis of plasmid DNA was performed with 10µl of newly prepared plasmid DNA combined with 10µl PCR mix (1 x *Taq* polymerase buffer, 2.5mM MgCl₂, 0.2mM dNTPs, 0.4µM each primer, 0.05units/µl *Taq* polymerase, sterile H₂O). Samples were run with the following PCR program 95°C, 1.5min followed by 30 cycles of denaturing (95°C, 1min) annealing (X°C, 1.5min) and chain extensions (72°C, 1min), where X is the annealing temperature of the primers used. An agarose gel was run of the PCR products and glycerol stocks were made of the positives by adding glycerol (0.5ml) to culture (0.5ml). These were vortexed and then placed at -80°C for long term storage.

2.3.17. DNA Sequencing Using the ABI 3100 System

The DNA to be analysed (approximately 1µg plasmid DNA) was combined with ABI BigDye® v3.1 (4µl), Buffer (200mM Tris pH9.0, 5mM MgCl₂) (4µl) (Web Scientific Ltd., Crewe, UK) and 3µM primer (2µl). A final volume of 20µl was achieved by the addition of sterile water. The reaction mix was placed in an Biometra® UNO-Thermoblock™ (Biometra, Glasgow, UK) using the programme of 96°C for 1min (initial denaturation), followed by 25 repeated cycles of 96°C for 30sec (denaturation), X°C for 15sec (annealing) and 60°C for 4min (extension). Labelled sequencing products were cleaned up using the Qiagen DyeEx 2.0 spin kit (Qiagen Ltd., West Sussex, UK). The DyeEx 2 spin column was vortexed and then the end snapped off and the column was placed in a 2ml collection tube. Column was centrifuged (2800rpm, 3min) then the column was transferred to a clean microcentrifuge tube. The sequencing reaction (20µl) was added dropwise to the centre of the gel bed in the column. After centrifugation (2800rpm, 3min) the column was discarded and the pelleted sample was dried in a vacuum centrifuge (60°C, 15min).

Sequence analysis was performed by either Steve Turner (DNA Sequencing Core, Cardiff University, UK) or Joyce Hoy and Barrie Francis (Central Biotechnology Services, Cardiff University, UK).

Chapter 3

RNAi induced gene silencing using siRNAs

3. RNAi induced gene silencing using siRNAs

3.1. Introduction

Induction of RNAi by double-stranded RNAs (dsRNAs) was found to produce small-interfering RNA (siRNA) duplexes as intermediates of the process (Zamore *et al.*, 2000). These are ~21 nucleotide (nt) sequences of RNA that have 2 nt 3' overhanging ends and are produced by the RNase III enzyme Dicer (Bernstein *et al.*, 2001). It was shown that a single strand of the siRNA duplex complexes with the RNA-induced silencing complex (RISC) resulting in the binding and cleavage of the homologous mRNA (Hammond *et al.*, 2000; Martinez *et al.*, 2002). Cleavage of the mRNA occurs in the centre of the region targeted by the siRNA between residues 10 and 11 (as counted from the 5' end) (Elbashir *et al.*, 2001b).

Transfections of siRNAs into *Drosophila S2* cells and mammalian cells were shown to induce RNAi in these cells (Elbashir *et al.*, 2001a). This allowed the use of RNAi for specific gene silencing in mammalian cells that were previously shown to have non-specific knock-down of gene expression upon transfection of dsRNA due to the interferon response. Studies have used either chemically synthesised siRNAs (Elbashir *et al.*, 2001a; Harborth *et al.*, 2001), those produced from recombinant Dicer (Carpenter and Zernicka-Goetz, 2004; Myers *et al.*, 2003) or T7 RNA polymerase produced siRNAs (Donze and Picard, 2002) for RNAi induction.

Chemically synthesised siRNAs consist of 19 bp with 2 nt 3' overhangs usually consisting of dTdT. This structure was found to be the most effective at gene silencing (Elbashir *et al.*, 2001c). The presence of the overhang has been shown to be important in efficient gene silencing (Elbashir *et al.*, 2001b) and has been proposed to help RISC recognition of an siRNA (Lingel *et al.*, 2004). Not all siRNAs induce gene silencing therefore several may need to be tested to induce specific gene silencing of the target mRNA.

An inexpensive and efficient method for producing siRNAs that target across the mRNA sequence is to use pools of siRNAs produced from recombinant Dicer. They are made by incubation of the dsRNA of the target gene with recombinant Dicer. These pools of siRNAs have been transfected into cells and shown to induce specific

gene silencing with no off-target effects observed (Carpenter and Zernicka-Goetz, 2004; Myers *et al.*, 2003).

In another study sense and antisense strands of siRNAs were transcribed from a T7 RNA polymerase promoter and subsequently annealed together to form siRNA duplexes. These T7 siRNAs were also shown to be effective mediators of RNAi induced gene silencing (Donze and Picard, 2002).

Here I report experiments designed to test the effectiveness of siRNAs to selectively reduce gene expression of both exogenously (*MmGFP*) and endogenously (*Oct-4*, *MmGFP*) expressed genes by means of RNAi in mES cells lines. It was determined that mES cell gene expression can be reduced by the transfection of siRNAs.

3.2. Experimental approach and results

siRNAs were designed against two target mRNAs, one encoding a modified form of green fluorescence protein (*MmGFP*) and the other *Oct-4*. *MmGFP* was targeted as it can be visualised by fluorescence microscopy and therefore its knockdown can be easily assessed. *Oct-4* is a member of the POU transcription factor family whose expression is downregulated in mES cells upon differentiation (Palmieri *et al.*, 1994). The level of *Oct-4* must be maintained with a certain range for ES cell self-renewal (Niwa *et al.*, 2000). A decrease in *Oct-4*, to less than 50% of normal levels, resulted in differentiation of mES cell to trophectoderm-like cells that express *Cdx2* and *Hand1*. These cells have distinct morphology as they 'flatten' out on the cell dish and therefore can be distinguished from undifferentiated mES cells. Therefore it was predicted that the transfection of *Oct-4* siRNAs would result in a reduction of *Oct-4* that could be assessed by microscopy and RT-PCR analysis.

3.2.1. Sequencing of *MmGFP*

The sequence of *MmGFP* has been reported (Siemering *et al.*, 1996; Zernicka-Goetz *et al.*, 1997) but it was important to verify the sequence in the pTP6 vector being used (Pratt *et al.*, 2000) (Appendix 1). Therefore primers GFP_F and R and *MmGFP*_F and R (Table 2.7) (Section 2.1.5) and the Big Dye v3.1 sequencing kit (Perkin Elmer Applied Biosystems, Warrington, UK) were used to determine the sequence *MmGFP*

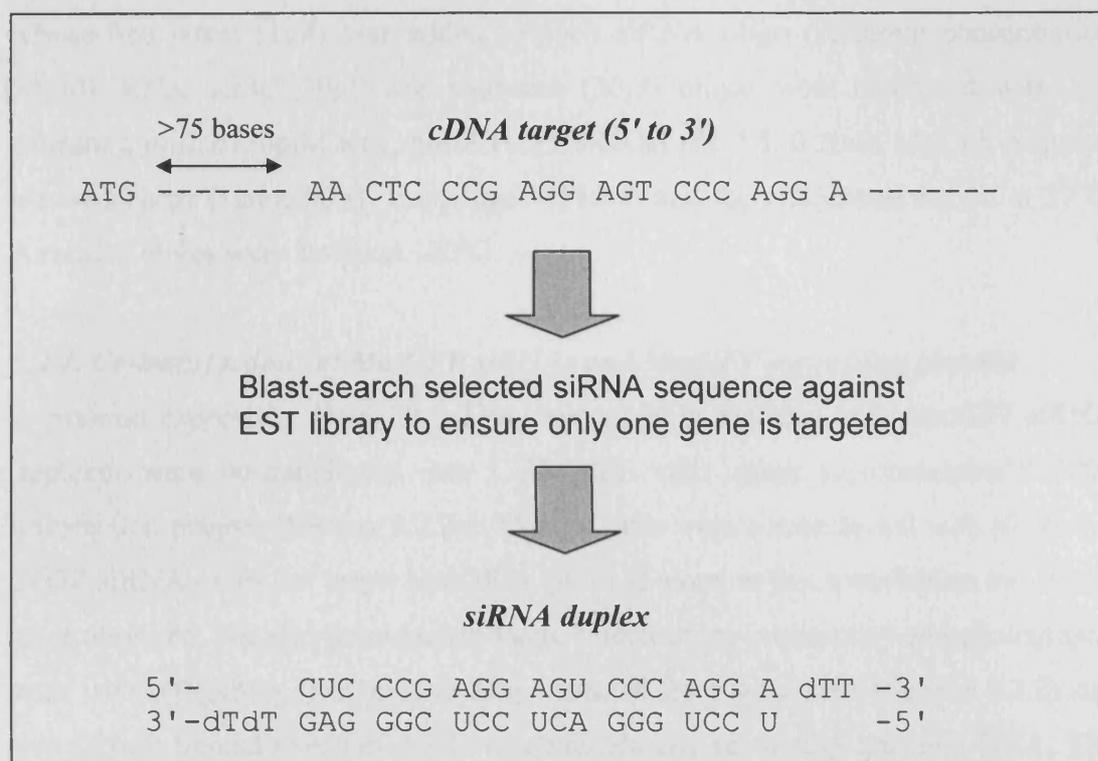


Figure 3.2. Designing siRNA oligos Start 50 to 100 nt from start site of cDNA sequence of gene to be targeted. Find an AA dimer and record the next 19 nt. Sequences that are AA (N19), where N is any nucleotide, with approximately 50% GC content should ideally be used. The siRNA oligos were synthesised with 2 nt 3' ends as these were found to be more efficient in reducing target RNA expression than blunt ended oligos (Elbashir *et al.*, 2002). [Figure adapted from Dharmacon Research technical bulletin #003, 2001 (Dharmacon Research, Lafayette, CO, USA)].

Targeted gene	Name of siRNA oligo	Sequence of siRNA oligonucleotides (5'-3')
<i>EGFP</i>	EGFP sense	CCACAUGAAGCAGCACGACdTdT
	EGFP antisense	GUCGUGCUGCUUCAUGUGGdTdT
<i>MmGFP</i> (Figure 3.1) (321-341nt)	MmGFP sense	GACACGUGCUGAAGUCAAGdTdT
	MmGFP antisense	CUUGACUUCAGCACGUGUCdTdT
<i>Oct-4</i> M34381 (374-392 nt)	Oct-4A sense	CUCCCGAGGAGUCCAGGAdTdT
	Oct-4A antisense	UCCUGGGACUCCUCGGGAGdTdT
<i>Oct-4</i> (621-639 nt)	Oct-4B sense	UGAGAACCUUCAGGAGAUAdTdT
	Oct-4B antisense	UAUCUCCUGAAGGUUCUCAdTdT
<i>Oct-4</i> (507-525 nt)	Oct-4C sense	AAAGGUGUUCAGCCAGACCdTdT
	Oct-4C antisense	GGUCUGGCUGAACACCUUUDdTdT

Table 3.1. List of siRNA oligos transfected into mES cells. Site of target within the gene is indicated as number of nucleotides from the start site in brackets.

3.2.3. Annealing of siRNA oligos

RNase-free water (1ml) was added to each siRNA oligo (resulting concentration 50 μ M). RNA sense (30 μ l) and antisense (30 μ l) oligos were combined with 1 x annealing buffer (20mM KCl, 6mM HEPES-KOH pH 7.5, 0.2mM MgCl₂). Solution was incubated (1min, 90°C) centrifuged (15sec) and then incubated for 1h at 37°C. Annealed oligos were stored at -20°C.

3.2.4. Co-transfections of *MmGFP* siRNAs and *MmGFP* expressing plasmid

A plasmid expressing *MmGFP* (pTP6, Appendix 1) together with *MmGFP* siRNA duplexes were co-transfected into CCB mES cells using Lipofectamine™ 2000 transfection reagent (Section 2.2.2a). Control cells were cotransfected with pTP6 and EGFP siRNA (does not target *MmGFP*), plasmid alone or just transfection mix. Cells were observed 72h after transfection using fluorescence microscopy and photographs were taken (Figure 3.3). RNA was also extracted from these cells (Section 2.3.2) and was DNase treated (Section 2.3.3) to eliminate any remaining genomic DNA. The RNA was then reverse transcribed to cDNA (Section 2.3.4). Semi-quantitative PCRs (Section 2.3.5) were performed to measure varying levels of *MmGFP* expression between different samples when cDNA had been normalised against the housekeeping gene, beta-actin (*Actb*). The primers used were Actb_F and R and *MmGFP*_F and R (Table 2.7).

GFP was seen in the cells that had been co-transfected with the pTP6 and EGFP siRNA and the cells that had only been transfected with pTP6 (Figure 3.3). There was no GFP observed in cells that had been co-transfected with *MmGFP* siRNA duplex and the pTP6 and those transfected with the transfection mix only. These results were confirmed by semi-quantitative RT-PCR looking at *MmGFP* expression levels comparing them to the house-keeping gene *Actb*. *MmGFP* was observed in cells cotransfected with the pTP6 plasmid and EGFP siRNA. This shows that the reduction in *MmGFP* observed is not due to the siRNA inhibiting the transfection of the pTP6 plasmid into cells.

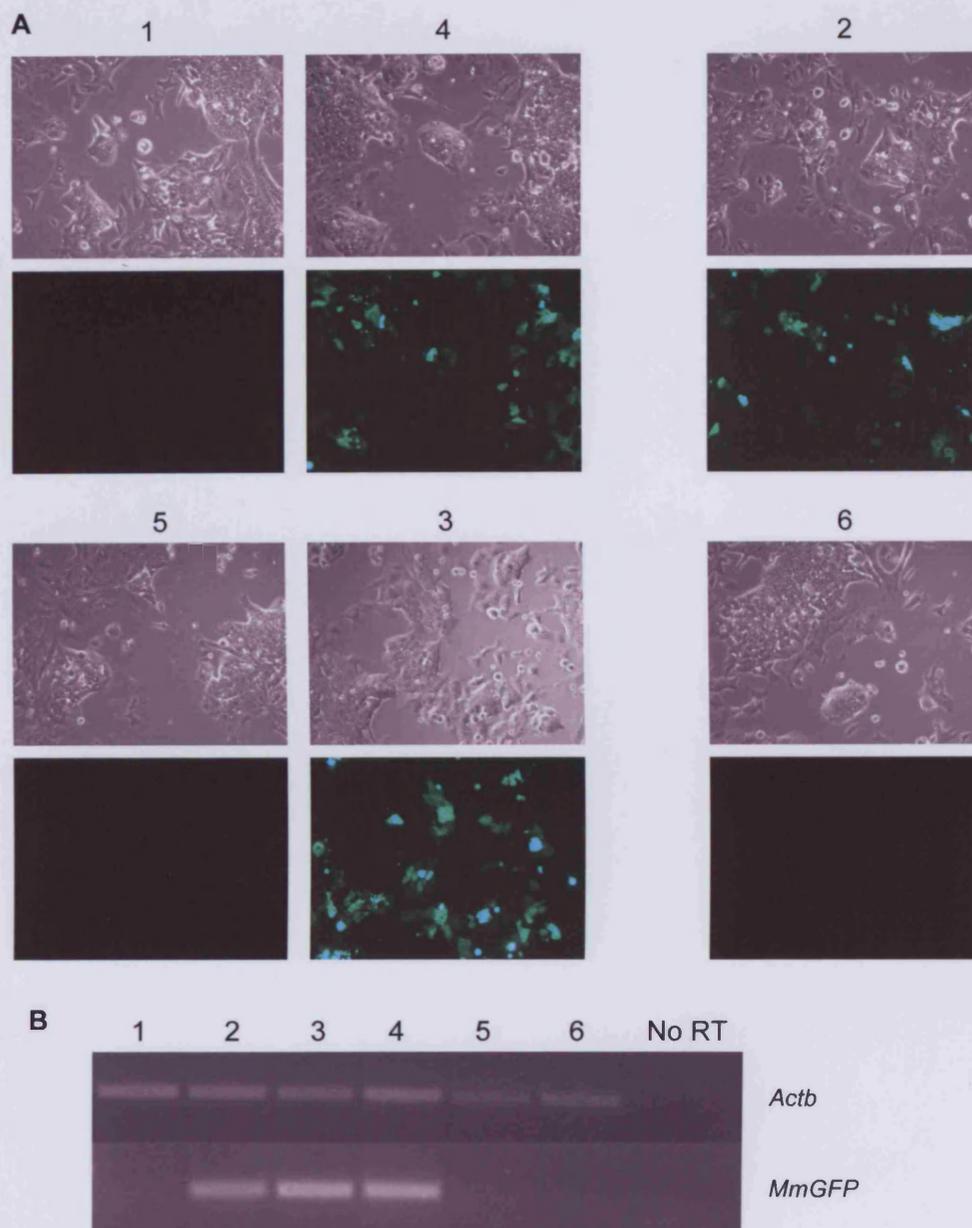


Figure 3.3. siRNA induced gene silencing of MmGFP in CCB cells. Cells were transfected with: 1) 1 μg pTP6, 1 μg MmGFP siRNA, 2) 1 μg pTP6 1 μg EGFP siRNA, 3) 2 μg pTP6 4) 1 μg pTP6. 5) 2 μg pTP6, 1 μg MmGFP siRNA 6) Transfection mix only. **A)** MmGFP expression was observed in control cells, that had been transfected with MmGFP expressing plasmid only (3 and 4) or cotransfected with the pTP6 vector and EGFP siRNA (2) that is not homologous to the MmGFP target. MmGFP was not observed in cells cotransfected with pTP6 and MmGFP siRNA. **B)** Semi-quantitative PCR confirmed these observations. No RT- a control reverse transcription of RNA sample 1 was set up without reverse transcriptase. This was to check for genomic DNA contamination. No PCR product was observed therefore confirming the PCR products that were observed are representative of the mRNA transcribed.

3.2.5. Knockdown of endogenously expressed *MmGFP* in mES cells

A mES cell line was made that expresses *MmGFP* constitutively so knockdown of a chromosomally expressed gene could be assessed. The pTP6 vector was transfected into ME2 mES cells (Table 2.4) using calcium phosphate transfection (Section 2.2.2b). Cells containing the vector were selected for by the addition of puromycin (3 μ g/ μ l). Individual colonies were picked and expression of *MmGFP* in the cells was confirmed by fluorescence microscopy. The cell line produced that was constitutively expressing *MmGFP* was named METP6.

METP6 cells were transfected with *MmGFP* siRNA using Lipofectamine™ 2000 transfection reagent (Section 2.2.2a). Cells were observed by fluorescence microscopy 72h post-transfection. There was a reduction in GFP in some cells within wells that had been transfected with *MmGFP* siRNA (Figure 3.4). There was no observable reduction in GFP in METP6 cells transfected with the EGFP siRNA duplex or just transfection mix showing that the reduction in GFP is due to the presence of *MmGFP* siRNA duplexes.

The difference in the knockdown of plasmid expressed *MmGFP* (Figure 3.3) and constitutively expressed *MmGFP* (Figure 3.4) was due to design of the two experiments. Following cotransfections of pTP6 and *MmGFP* siRNA into CCB cells (Figure 3.3) it is predicted cells transfected with the *MmGFP* expressing plasmid also contain the siRNA duplex. Therefore no *MmGFP* was observed as it was knocked down in all transfected cells. In the METP6 cells (Figure 3.4) all of the cells express *MmGFP* but not all of the cells were transfected with the *MmGFP* siRNA. Therefore fewer cells with *MmGFP* knockdown were seen.

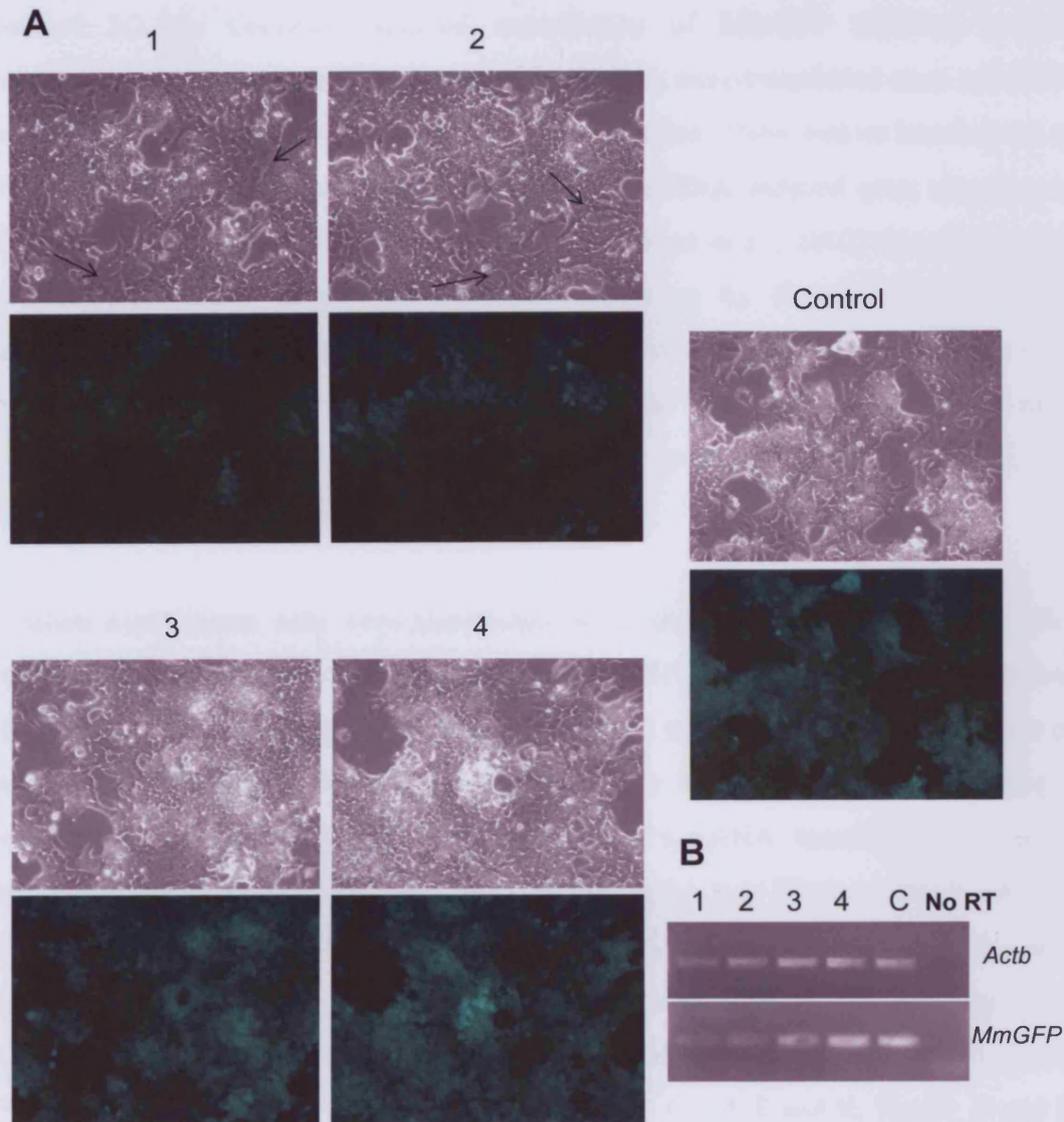


Figure 3.4. METP6 ES cells transfected with MmGFP siRNAs A) Photographs of cells transfected with: **1)** MmGFP siRNA (1 μ g), **2)** MmGFP siRNA (2 μ g), **3)** EGFP (1 μ g), **4)** EGFP (2 μ g), **C** Transfection medium only. The arrows indicate regions of cells with MmGFP knockdown. There was no reduction in MmGFP expression in the control experiments (3-5). **B)** A reduction in *MmGFP* in MmGFP siRNA transfected cells (1 & 2) was confirmed by semi-quantitative RT-PCR.

3.2.6. Knockdown of *Oct-4* in mES cells

Oct-4 siRNA duplexes (Table 3.1) were transfected into METP6 mES cells that had been seeded into 24-well plates using Lipofectamine™ 2000 transfection reagent (Section 2.2.2a). Controls included transfection of MmGFP siRNAs, or just transfection mix. In my preliminary experiments cells were transfected once and RNA extracted for RT-PCR analysis 72h after the transfection. There was no knockdown of *Oct-4* observed (results not shown). The effect of siRNA induced gene silencing in mammalian cells was shown to be transient (McManus *et al.*, 2002) therefore it was predicted that *Oct-4* expression may be recovering by the time analysis was performed. ES cells divide every 12 to 20h whereas once trophectoderm cells are fully committed they do not divide at all. Therefore at the 72h time point cells that have differentiated due to *Oct-4* knockdown will be out grown by the untransfected, and therefore undifferentiated ES cells.

In future experiments cells were transfected twice, the second transfection 24h after the first. Photographs of the cells were taken and RNA extracted 72h after the first transfection. The presence of flattened, differentiated cells was observed in cultures of Oct-4A and Oct-4B siRNA transfected cells (Figure 3.5A). There was no change in morphology of control cells (transfected with METP6 siRNA, transfection mix only) or Oct-4C siRNA transfected cells. The extracted RNA was DNase treated and then reverse-transcribed as described (Section 3.2.4). Semi-quantitative PCR was performed on the cDNA to study gene expression of *Actb* (house keeping gene, control), *Oct-4* (gene of interest), *Cdx2* and *Hand1* (markers of trophectoderm) (Figure 3.5). The primers used were *Actb*_F and R, *Oct-4*_F and R, *Hand1*_F and R and *Cdx2*_F and R as listed in Table 2.7.

It was expected that METP6 cells that had been transfected with any of the *Oct-4* siRNA duplexes (Figure 3.5 #2, 3, 4, 5) would have a reduction in *Oct-4* expression as compared to control cells (Figure 3.5 #1, 6). A small reduction in *Oct-4* was seen by semi-quantitative PCR (Figure 3.5B) in cells transfected with Oct-4A and Oct-4B siRNAs but not in cells transfected with Oct-4C siRNA only. A reduction in *Oct-4* expression has been shown to induce differentiation of mES cells to trophectoderm cells that express *Cdx2* and *Hand1* (Niwa *et al.*, 2000).

Chapter 3: RNAi induced gene silencing using siRNAs

CDX2 was found to be present at day 3.5 of mouse embryonic development in the trophectoderm but was absent from the inner cell mass (Beck *et al.*, 1995). *Hand1* (eHand) was also shown to be expressed at high levels in trophoblast cells (Cserjesi *et al.*, 1995). *Hand1* and *Cdx2* were subsequently confirmed as markers of trophectoderm by studying their gene expression in trophoblast stem cells derived from differentiated ES cells (Niwa *et al.*, 2000). Therefore the increase in expression of *Cdx2* and *Hand1* observed (Figure 3.5B) in Oct-4A and Oct-4B siRNA transfected cells shows their differentiation down the trophectoderm lineage.

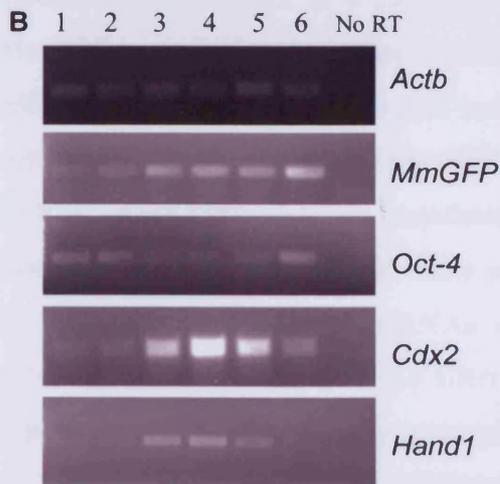
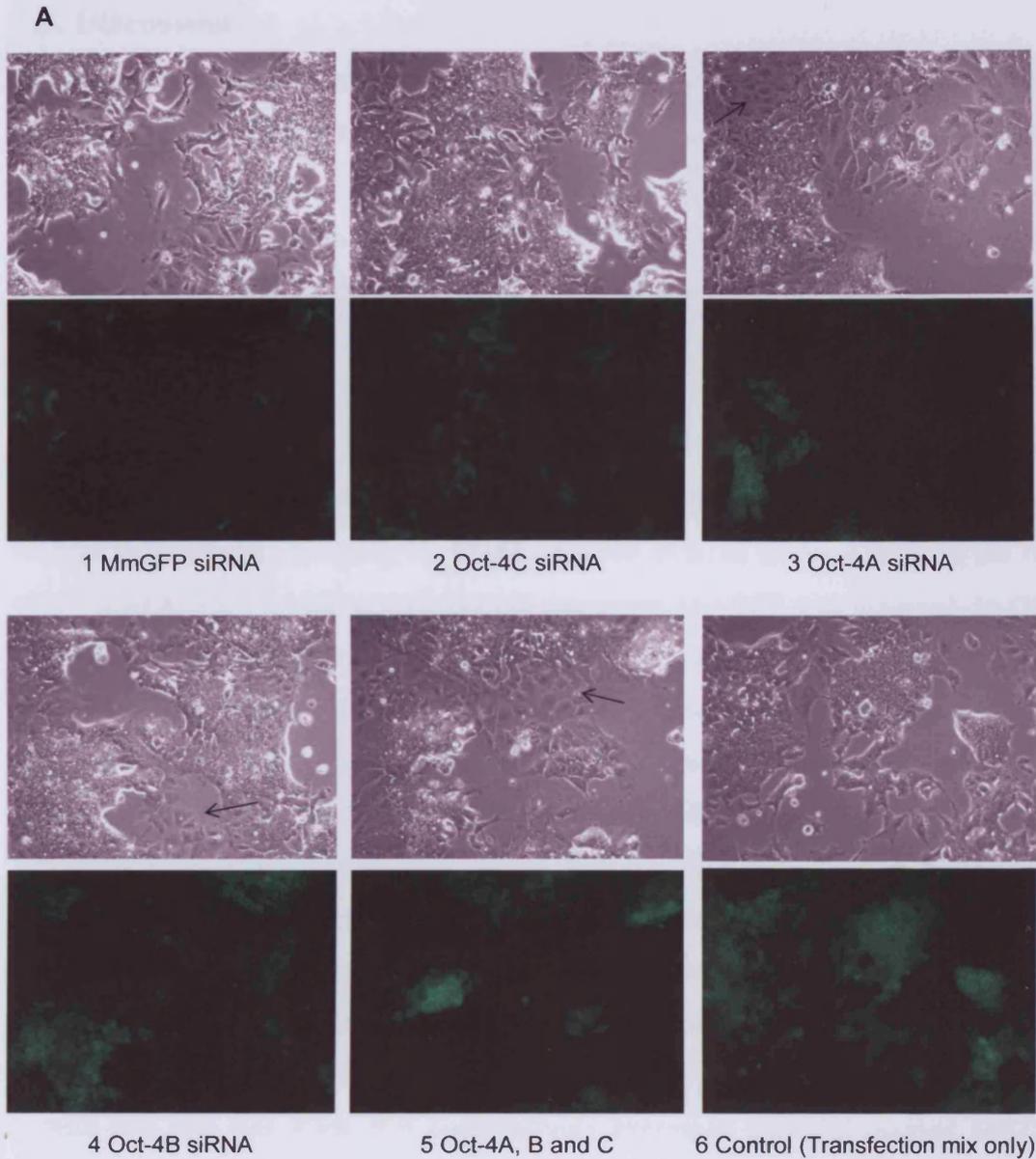


Figure 3.5 Oct-4 knockdown in METP6 cells. **A)** Morphology of siRNA transfected cells. The arrows indicate examples of flattened differentiated cells. **B)** There was a reduction in the expression of *Oct-4* compared to *Actb* in cells that been transfected with Oct-4A and B (#3, 4, and 5) duplexes. This corresponded to an increase in the expression of trophoctoderm marker *Cdx2* and *Hand1*. There was no reduction of *Oct-4* in Oct-4C siRNA transfected cells or increase in trophoctoderm marker expression.

3.3. Discussion

Since their discovery as intermediates in the processing of dsRNA to cause RNAi, chemically or recombinant Dicer produced siRNAs and T7 polymerase produced siRNAs have been shown to be effective for gene silencing (Caplen *et al.*, 2001; Carpenter and Zernicka-Goetz, 2004; Donze and Picard, 2002; Elbashir *et al.*, 2001a; Harborth *et al.*, 2001; Myers *et al.*, 2003). Transfection of siRNAs is preferential to dsRNA for induction of RNAi in most mammalian cell lines as the interferon response is not initiated by these shorter RNA duplexes (Harborth *et al.*, 2001).

In the study described here chemically synthesised siRNAs were used to establish whether gene specific silencing by RNAi could be induced in these cells. In the first set of experiments knockdown of plasmid expressed MmGFP was assessed. In CCB mES cells that were cotransfected with the MmGFP expressing plasmid (pTP6) and MmGFP siRNA duplexes no GFP was observed 72h post transfection. In contrast to control cells that were only transfected with pTP6 (Figure 3.3). The reduction in GFP was shown not to be just due to the presence of the siRNA as no reduction in GFP was observed in cells cotransfected with pTP6 and a siRNAs that is not homologous to *MmGFP* (EGFP siRNA). These experiments confirmed that the MmGFP siRNA designed reduces expression of the plasmid expressed *MmGFP*. The next step was to investigate knockdown of chromosomally integrated *MmGFP*.

A mES cell line was made that constitutively expresses *MmGFP* (named METP6 cells) and then these cells were transfected with MmGFP siRNA oligos. There was a knockdown of MmGFP in some cells (Figure 3.4) whereas there was no reduction in MmGFP in control cells (either transfected with just transfection mix or with EGFP siRNA). Not all of the cells would have been transfected therefore it is possible the cells that have knockdown of MmGFP have taken up the MmGFP siRNA and those with no knockdown were not transfected. Alternatively the MmGFP siRNA may not have induced gene silencing in these cells. This could be tested in future studies by the transfection of labelled siRNAs so that a knockdown effect can be directly attributed to the presence of the siRNA in the cell. These studies showed that the expression of a chromosomally integrated gene can be reduced using siRNAs in these mES cells. The use of labelled siRNAs may allow either the enrichment of transfected

cells by using fluorescence activated cell sorting (FACS) or allow cell by cell assay for studying a knockdown effect of a particular gene on the phenotype of the cell.

Oct-4 was chosen as a second gene for RNAi targeting. It is a transcription factor whose expression is important in maintaining the pluripotency of mES cells (Palmieri *et al.*, 1994). Reduction of *Oct-4* in ES cells results in their differentiation to trophectoderm (Niwa *et al.*, 2000). Three siRNAs for *Oct-4* were designed using the method as described by Harborth *et al.* (Harborth *et al.*, 2001) (Figure 3.1). These siRNA duplexes were transfected into METP6 ES cells and gene expression of the cells was looked at. A reduction in *Oct-4* was seen in cells transfected with Oct-4 siRNAs A and B (Figure 3.4B) and also corresponded with an increase in expression of the trophectoderm marker genes *Cdx2* and *Hand1*.

Since these experiments were performed a study has been published that compared the role of Oct-4 in mES and human ES (hES) cells by transfection of siRNAs (Hay *et al.*, 2004). Analysis of gene expression at 24h, 48h and 72h post transfection showed *Oct-4* to be reduced by 24h and cells were observed to 'flatten' after 24h and by 48h most colonies were seen to be composed of 'overtly differentiated cells'. Also as in my study an increase in *Cdx2* expression was observed. The authors also noted an upregulation of the endodermal marker genes *Gata6* and *α -fetoprotein*. The expression of these genes was not looked in my study but this could be confirmed by further PCR analysis of my samples.

Transfection of Oct-4C siRNA did not result in a reduction in *Oct-4* expression or increase in *Hand1* and *Cdx2*. This may be explained in the light of information now known about the RNAi mechanism and the characteristics of functional siRNAs. In siRNA induced silencing a single strand of the siRNA is incorporated into the RNA-induced silencing complex (RISC) and the other strand is degraded (Martinez *et al.*, 2002; Schwarz *et al.*, 2003). The activated RISC then binds the mRNA that is homologous to the incorporated siRNA resulting in its cleavage. The strand of the siRNA duplex that is retained by RISC has been shown to be the strand with the lowest internal stability at its 5' end (Schwarz *et al.*, 2003). This was supported by other studies into the features of functional siRNAs all of which showed that the 5' end of the antisense strand should have a lower internal stability than the 5' end of the

sense strand (Khvorova *et al.*, 2003; Reynolds *et al.*, 2004; Schwarz *et al.*, 2003; Ui-Tei *et al.*, 2004). This is predicted to be because the lower internal stability aids unwinding of the siRNA duplex. In the study by Khvorova *et al.* it was also found that the region of 9-14bp (starting from the 5' antisense strand) had a low internal stability (Khvorova *et al.*, 2003). This region is around the site of mRNA cleavage and the low internal stability is predicted to allow quick release of the cleaved target mRNA. Therefore the RISC still complexed with the siRNA is able to go and seek new mRNAs. Contrary to this another study found no reduction in the absolute values of delta G° in the region 9-14 (Ui-Tei *et al.*, 2004).

In another study (Reynolds *et al.*, 2004) looked at the features that were present in functional siRNAs and absent from non-functional siRNAs. The efficiency of 180 siRNAs to reduce the expression of two genes was examined. Eight characteristics were determined from the analyses which are important in order for a siRNA to be functional. An algorithm was constructed using these for designing functional siRNAs that can induce gene specific silencing (Table 3.2). They used this criteria for designing siRNAs and 29 out of 30 of those tested induced more than 50% silencing which was a 3.5 fold improvement compared to using randomly selected siRNAs for the same genes.

Characteristic	Points
G/C content (36-52%)	1
Presence of A/U at positions 15-19 of the sense strand	1 point for each A or U base present
No internal repeats (T _m value <20°C)	1
Presence of A at position 19 of the sense strand	1
Presence of A at position 3 of the sense strand	1
Presence of U at position 10 of the sense strand	1
Presence of A or T at position 19 of sense strand	-1
Presence of C, A, or T at position 13 of sense strand	-1

Table 3.2. Scoring system for determining functional siRNAs (Reynolds *et al.*, 2004). Maximum score is 10 and a functional siRNA was defined as having a score of 6 points or more.

Software has been developed to assist in the designing of functional siRNAs using the criteria discussed above. One such program Sfold, available on the internet (<http://sfold.wadsworth.org>) was used to determine whether Oct-4C siRNA inability to induce RNAi could be explained. The software calculates the stability of siRNA 5' sense and antisense strands (end 4 base pairs) to allow the calculation of differential stability of the siRNA duplex ends (DSSE) (Ding *et al.*, 2004). In order for a siRNA duplex to be functional it must have a DSSE value greater than 0. The DSSE values for Oct-4 siRNAs were calculated to be 1.7, 2.1, and -4.4 kcal/mol for A, B and C respectively (Table 3.3). The sense strand of the Oct-4C siRNA duplex has a lower 5' end stability than the 5' end of the antisense strand. Therefore the sense strand of Oct-4C siRNA would have been loaded into RISC. The antisense strand that is homologous to the target sequence would be degraded. Hence, there was no reduction of Oct-4 expression by transfection of this siRNA duplex (Figure 3.5).

The siRNA were designed using the available literature at the time (Elbashir *et al.*, 2001a; Elbashir *et al.*, 2002). It was recommended that the target sequence should begin with an AA, and ideally have the sequence 5'-AA(N₁₉)UU (where N is any nucleotide) in the mRNA. The sequence should also have a GC content of ideally of 50% (but <70% and >30%) and avoid highly rich GC regions. Sequences were Blast searched (<http://www.ncbi.nlm.nih.gov/BLAST/>) against mouse EST libraries to ensure only one gene is targeted. The information that is now known about functional siRNAs has resulted in more detailed criteria for designing siRNAs.

The total siRNA duplex score in Sfold is calculated from the sum of the target accessibility score, duplex feature score and duplex thermodynamics score and has a maximum value of 20. A score of 12 or greater predicts a functional siRNA. The target accessibility score is calculated from the RNA/RNA stacking energies in the antisense siRNA and target sequence hybrid (Ding and Lawrence, 2001; Ding and Lawrence, 2003) (scores between 0 and 8). Duplex feature score (range -2 to 10 points) was calculated using the algorithm designed by Reynolds *et al.* (Reynolds *et al.*, 2004) as described in Table 3.2. The duplex thermodynamics score was made up of 1 point if DSSE was greater than 0 and 1 point if the average of the internal stability values for positions 9-14 was greater than -8.6kcal/mol. Oct-4C gave a total siRNA duplex score of 11 that is below the 12 points or more needed for a functional

siRNA. Therefore using the rules determined by the Sfold software Oct-4C is predicted not to induce gene silencing as was shown in these experiments (Figure 3.5).

siRNA	Target accessibility score	Duplex Feature score	5' AS stability (AS) (kcal/mol)	5' sense stability (SS) (kcal/mol)	DSSE (AS – SS) (kcal/mol)	AIS (kcal/mol)	siRNA duplex score
Oct-4A	8	4	-10.0	-11.7	1.7	-10.6	13
Oct-4B	8	8	-7.0	-9.1	2.1	-8.3	18
Oct-4C	7	3	-11.2	-6.8	-4.4	-8.4	11
MmGFP	8	4	-8.1	-10.1	2.0	-9.4	13

Table 3.3. Calculation of the internal stability of the siRNAs using ‘Sfold’ (Ding *et al.*, 2004) (<http://sfold.wadsworth.org>) It has been shown that functional siRNA duplexes normally have lower stability at the 5' end of the antisense strand than at the 5' end of the sense strand (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). The differential stability of the siRNA duplex ends (DSSE) was calculated and is a measure of this. A functional siRNA duplex has a DSSE score of greater than 0. The total duplex score is calculated from the target accessibility score, the duplex feature score and duplex thermodynamics score. A functional siRNA should have a total siRNA duplex score of 12 or more.

Since these experiments were performed, an optimised method for transfection of siRNAs into mES cells using Lipofectamine™ 2000 was published (Takada *et al.*, 2005). In this method mES cells were removed from the bottom of the culture plate on the day of transfection. Lipofectamine 2000/siRNA mix was added to the cells in suspension. The study knocked down *EGFP* and *Oct-4*. They observed greatest knockdown of *Oct-4* expression at 24h post-transfection after which time the *Oct-4* began to return to normal levels. The expression of *Hand1* was observed from 48h post transfection. The use of this transfection protocol may increase the number of mES cells transfected thereby allowing effects of knockdown to be studied more easily. In future siRNA experiments gene expression analysis will be performed on RNA samples extracted over a time course. Therefore the time of maximum knockdown and any ‘recovery’ of target gene expression can be observed.

Methods to generate several siRNAs targeting the same gene to increase the likelihood of knockdown by siRNAs have been developed. Yang and colleagues used an *E.coli* RNase III enzyme to cleave dsRNA into short siRNAs referred to as endoribonuclease-prepared siRNA (esiRNA) (Yang *et al.*, 2002). The generation of

Chapter 3: RNAi induced gene silencing using siRNAs

pools of esiRNAs allows the gene of interest to be targeted more effectively as multiple sites within an mRNA are targeted simultaneously. In this study cross-target gene silencing was not observed.

Carpenter and Zernicka-Goetz used Dicer produced pools of siRNAs (dsiRNAs) from dsRNA to knockdown *Oct-4* in ES cells leading to their differentiation to trophectoderm (Carpenter and Zernicka-Goetz, 2004). DsRNA targeting *Oct-4* was produced by *in vitro* transcription of cDNA then siRNAs were produced by digestion of the dsRNA with the recombinant human ribonuclease III enzyme, Dicer. These diced siRNAs were transfected into cells using Lipofectamine™ 2000. Knockdown of *Oct-4* led to differentiation of ES cells to trophectoderm as shown by increase in expression of *Cdx2* and *Hand1* assessed by RT-PCR 24h post-transfection. They observed 80% transfection efficiency when FITC labelled siRNAs were analysed by fluorescence activated cell sorting (FACS) analysis. Dicer produced pools of siRNAs target across the mRNA therefore increasing the likelihood of knockdown of the target mRNA as the pool would be expected to contain some functional siRNAs.

Transfection of siRNAs into mES cells has been shown to induce gene specific silencing. Limitations for using this method for inducing RNAi include that not all siRNAs are effective at silencing and that their delivery is inefficient in mES cells. Increasing understanding of the mechanism of RNAi and the features present in functional siRNAs is resulting in improvements to the design of functional siRNAs. Also improved transfection methods have been published that would be tested in future experiments (Takada *et al.*, 2005). However, even overcoming these limitations the knockdown induced by transfected siRNAs is transient therefore long term effects of gene silencing cannot be studied. Vectors expressing short-hairpin RNA (shRNA) have been developed to overcome this problem. These vectors can result in stable gene silencing as the shRNA, that is the RNAi trigger, is continually expressed in cell lines stably transfected with the shRNA vector.

Chapter 4

RNAi induced gene silencing using vectors expressing shRNAs

4. RNAi induced gene silencing using vectors expressing shRNAs

4.1. Introduction

Vectors expressing short hairpin (sh) RNAs from RNA polymerase III promoters can be used for inducing stable knockdown of gene expression by RNAi. Expressed shRNAs fold to produce RNAs with ~21bp stems that are joined by a 6-8nt loop. These shRNAs are processed by Dicer *in vivo* to mature siRNAs that can induce gene specific silencing (Paddison *et al.*, 2002).

RNA polymerase III promoters that have been used for expression of shRNAs include the human H1 RNA promoter (Brummelkamp *et al.*, 2002; Kunath *et al.*, 2003), mouse U6 promoter (Sui *et al.*, 2002; Tang *et al.*, 2004) and the human 7S K promoter (Koper-Emde *et al.*, 2004). These are effective promoters for transcription of shRNAs as they are small (100, 250 and 250bp respectively), the RNA these promoters transcribe has a definite start site and a termination of transcription signal consisting of 4 or 5 consecutive thymidines (Tuschl, 2002). The cleavage of the RNA occurs after the second uridine when reaching the termination site resulting in the production of shRNAs that have the characteristic overhanging ends observed in siRNAs (Elbashir *et al.*, 2001b).

In this study two vectors were used that contain the human H1 polymerase promoter from which shRNAs are transcribed. The genes that were targeted for knockdown were *Laminin B1*, *Rex-1* and *Oct-4*.

Rex-1 (*Zfp-42*) encodes an acidic zinc finger protein which is involved in regulating the transcription of developmental genes. *Rex-1* mRNA is found in undifferentiated mES and EC cells, in inner cell mass of the blastocyst and in trophectoderm (Rogers *et al.*, 1991). *Rex-1* is downregulated upon differentiation of the ICM into embryonic ectoderm and differentiation of mES cells. It was predicted that *Rex-1* knockdown would induce differentiation of the mES cells as it is a transcription factor (Ben-Shushan *et al.*, 1998) and its expression is down-regulated upon differentiation of mES cells (Rogers *et al.*, 1991).

Laminin B1 (*Lamb1*) is part of a basement specific glycoprotein laminin 1 (Sasaki *et al.*, 1987). Laminin 1 is expressed at the early stages of embryogenesis and is made up of the laminin chains $\alpha 1$, $\beta 1$, $\gamma 1$ (Colognato and Yurchenco, 2000). *Lamb1* was chosen as a target as it was predicted the reduction in expression of this gene would not affect the viability of the mES cells. Laminin B1 knockout mice have been made using gene trapping (Mitchell *et al.*, 2001) and function of *Lamb* was studied in embryos (Miner *et al.*, 2004). *Lamb1*^{-/-} embryos do not have basement membranes and only survive up to embryonic day 5.5.

4.2. Experimental Approach and Results

4.2.1. psiRNA-hH1zeo

The first vector used for shRNA induced gene silencing was the psiRNA-hH1zeo (psiRNA) vector that contains the human H1 RNA polymerase III promoter (Autogen Bioclear, UK Ltd., Wiltshire, UK) (Figure 4.1). This vector was one of the first commercially available shRNA expressing vectors and contained Zeocin™ selection. A region of each of the target genes, *Lamb1* and *Rex-1*, was selected according to the instructions received with the vector. The targeted sequence had to begin with an A (start site of H1 promoter), and sequences containing 4 or 5 consecutive Ts (that acts as termination signal of the polymerase III) were avoided. Oligonucleotides were designed to contain the target sequence as an inverted repeat separated by a 5 base spacer region that when annealed (Section 2.3.9) formed duplexes with *BbsI* overhanging ends (Table 4.1; Figure 4.2) for cloning into the psiRNA vector.

The annealed oligos were ligated into the *BbsI* digested vector (Section 2.3.13) and then transformed into *E. coli* GT116 bacterial strain (Section 2.3.15). This strain of bacteria is a *sbcCD* deletion strain. SbcCD is a protein complex that recognises and cleaves hairpins (Connelly *et al.*, 1998). Therefore this *E. coli* GT116 strain was used to increase the amount of recombinant clones that contain the plasmid with the hairpin insert. Growth of bacteria on LB agar plates containing Zeocin™ allowed the selection of bacteria that were transformed with the psiRNA plasmid. X-gal was also present in the agar and allowed distinction between bacteria containing the plasmid with the inserts (white colonies, as β -galactosidase is no longer expressed) compared

to those without the insert [blue colonies as β -galactosidase is expressed and cleaves X-gal (5-bromo-4-chloro3-indolyl- β -galactopyranoside) into glucose and a blue insoluble product]. Plasmid DNA was prepared from the 'white' colonies (Section 2.3.6a) and the presence of the insert was confirmed by *NcoI* digest (Section 2.3.10a) (Figure 4.2) and then sequence verified using primer OL381 (Section 2.3.17) (Table 2.7).

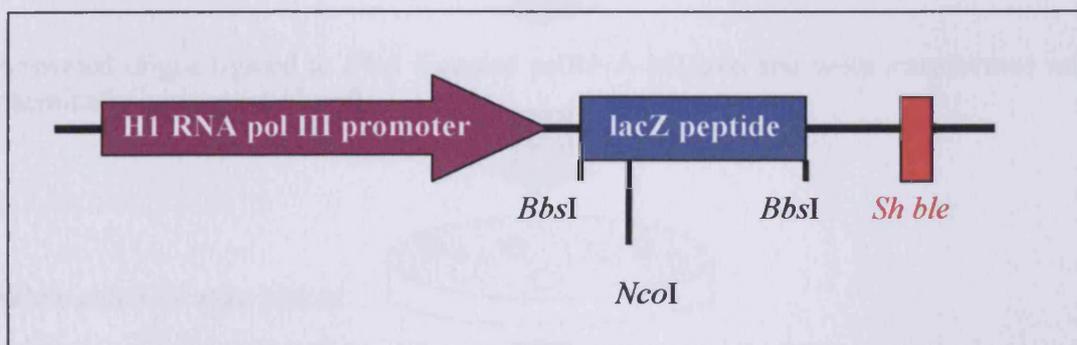


Figure 4.1. Main features of the psiRNA-hH1zeo. The vector contains the H1 RNA polymerase III promoter. Annealed oligos are cloned into the *BbsI* sites of the plasmid. Insertion of the oligos replaces *lacZ*. Therefore colonies containing the insert were identified as white colonies on LB agar plates containing X-gal. Presence of the insert was confirmed by *NcoI* restriction digest. Expression of *Sh ble* confers resistance to the antibiotic Zeocin™ allowing selection of transfected cells containing the vector.

Oligonucleotide	Sequence (5'-3')
Lamb1 si S	TCCCATGACATCATCTGTGACTCTCCATTGAGAGTCACAGAT GATGTCATTT
Lamb1 si AS	CAAAAAATGACATCATCTGTGACTCTCAATGGGAGAGTCACAG ATGATGTCAT
Rex-1 si S	TCCCATAGAGTGAGTGTGCAGTGCACCATTTGCACTGCACACT CACTCTATTT
Rex-1 si AS	CAAAAAATAGAGTGAGTGTGCAGTGCAAATGGTGCCTGCACA CTCACTCTAT

Table 4.1. Sequences of oligonucleotides that were annealed together and subsequently cloned into psihH1zeoRNA vector (Autogen-Bioclear, Wiltshire, UK). Highlighted region corresponds to target region of mRNA. (All oligos were purchased from MWG, Milton Keynes, UK).

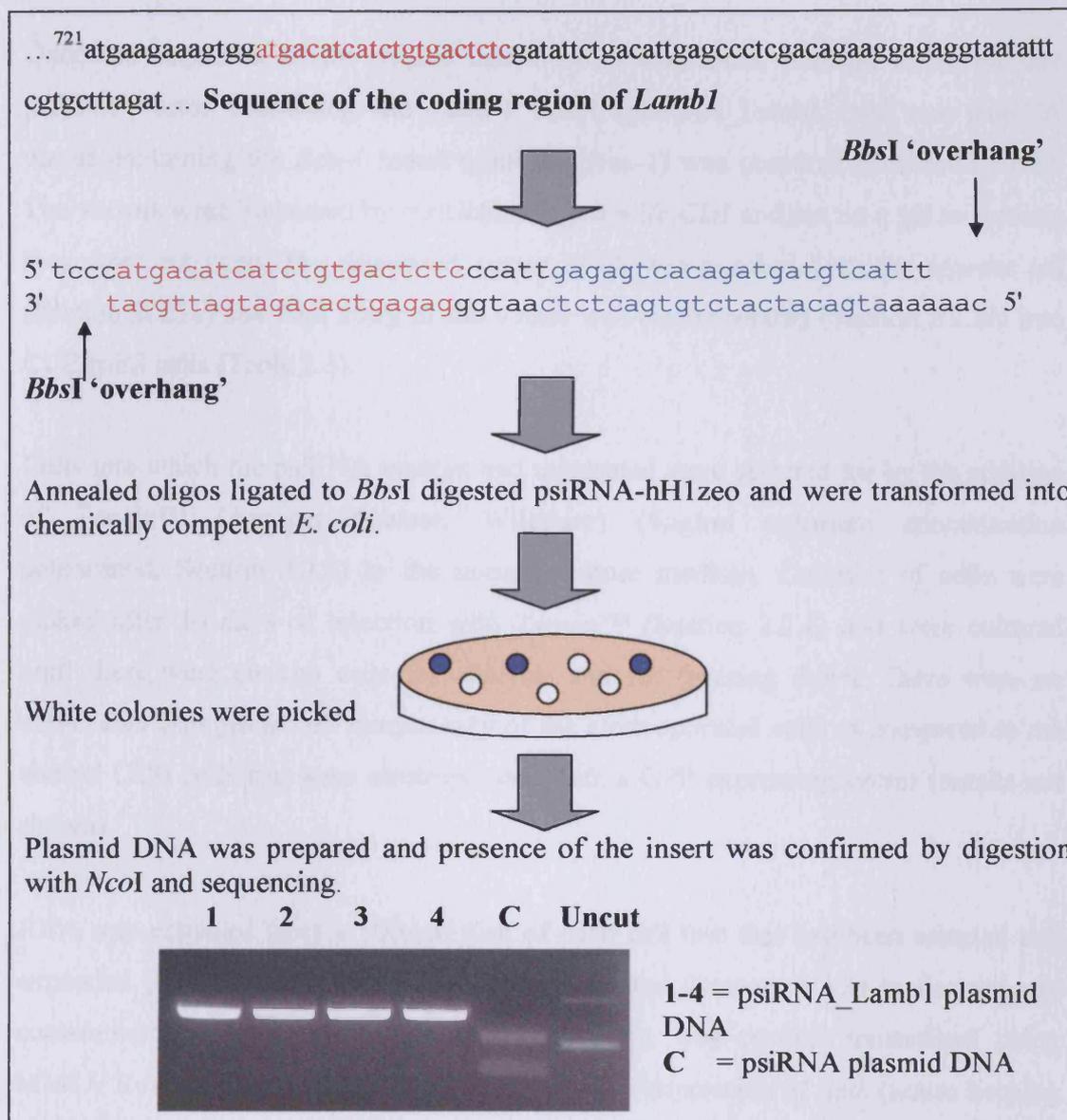


Figure 4.2. Process of cloning inverted repeat of gene of interest in psiRNA-hH1zeo. A 22 nt sequence from the coding region of the target, *Lamb1*, was selected according to the instructions received with the vector (Autogen Bioclear, UK Ltd., Wiltshire, UK). The targeted sequence started with an A (start site of H1 promoter), and avoided sequences containing 4 or 5 consecutive Ts (as acts as termination signal of the polymerase III). Oligonucleotides were designed to contain the target sequence as an inverted repeat separated by a 5 base spacer region and that when annealed formed duplexes with *BbsI* overhanging ends. The annealed oligos were ligated into the *BbsI* digested psiRNA-hH1zeo (psiRNA) vector (Autogen-Bioclear, Wiltshire, UK) and then transformed into *E. coli* GT116 cells. Growth of bacteria on LB agar plates containing Zeocin™ and X-gal allowed the selection of bacteria that contained the plasmid. White colonies were selected as *lacZ* is no longer expressed when an insert is present. Colonies were picked and cultured overnight and plasmid DNA extracted from the bacteria. The present of the insert was confirmed by *NcoI*, parental psiRNA-hH1zeo (C) results in the production of two fragments whereas when the insert is present the plasmid is linearised (1-4).

Chapter 4: RNA induced gene silencing using vectors expressing shRNAs

Once the sequence of the vector inserts were confirmed, plasmid DNA for one psiRNA vector containing the *Lamb1* insert (psiRNA_*Lamb1*) and one psiRNA vector containing the *Rex-1* insert (psiRNA_*Rex-1*) was prepared (Section 2.3.6.b). The vectors were linearised by restriction digest with *ClaI* and run on a gel to confirm they were cut once. The linearised vector DNA was purified from the agarose gel (Section 2.3.11) and then 20µg of the vector was electroporated (Section 2.2.2c) into CCB mES cells (Table 2.3).

Cells into which the psiRNA vectors had integrated were selected for by the addition of Zeocin™ (Autogen-Bioclear, Wiltshire) (5µg/ml optimum concentration determined, Section 2.2.3) to the normal culture medium. Colonies of cells were picked after 10 days of selection with Zeocin™ (Section 2.2.4) and were cultured until there were enough cells for analysis and for freezing down. There were no observable changes in cell morphology of the electroporated cells as compared to the control CCB cells that were electroporated with a GFP expressing vector (results not shown).

RNA was extracted from a 100mm dish of each cell line that had been selected and expanded (Section 2.3.2). RNA was DNase treated (Section 2.3.3) to destroy any contaminating genomic DNA present, then RNA was reverse transcribed using MMLV Reverse Transcriptase (Section 2.3.4). The expression of *Actb* (house keeping gene), *Lamb1* and *Rex-1* were analysed by semi-quantitative PCR (Section 2.3.5) (Figure 4.3) using primers *Actb_F* and R, *Lamb1_F* and R and *Rex-1_F* and R respectively (Table 2.7).

There was only a reduction in *Rex-1* expression in one of the CCB psiRNA_*Rex-1* cell lines tested (#14) (Figure 4.3A) whereas there was a reduction in *Lamb1* in all the cell line tested compared to the control (cDNA extracted from untransfected CCBs) (Figure 4.3B). PCRs were performed from genomic DNA (gDNA) extracted from CCB psiRNA_*Rex-1* cell lines #14-18 using psiRNA primers (Table 2.7) specific for the psiRNA vector (Figure 4.4). This was to confirm integration of the vector in cell line #14 and look for presence of the vector in other cell lines. It was expected that there would be a PCR product in CCB psiRNA_*Rex-1* cell line #14 corresponding to the vector and that #15, 16 and 17 would give no PCR product as these plasmids did

Chapter 4: RNA induced gene silencing using vectors expressing shRNAs

not demonstrate *Rex-1* knockdown. PCR products were shown in cell lines #14 and #16 showing that the vector had integrated into these cells. The psiRNA vector in #16 may have integrated into inactivated DNA and therefore was not expressing the *Rex-1* shRNA explaining why no knockdown of *Rex-1* was observed (Figure 4.3A). A reduction in *Rex-1* is expected to interfere with the ES cell phenotype and may explain why less cell lines with *Rex-1* knockdown were established. A reduction of *Lamb1* is not predicted to be critical to ES cell self renewal and so more cell lines with knockdown of *Lamb1* were established.

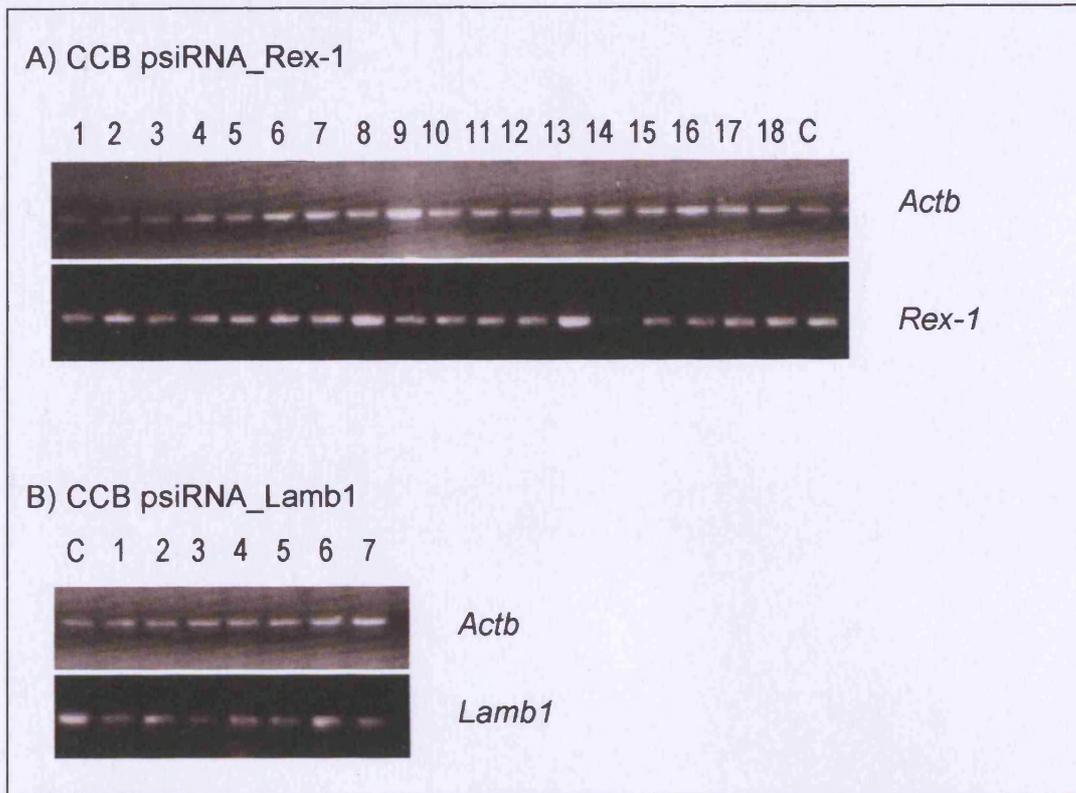


Figure 4.3. Results of PCRs of cDNA extracted from CCB mES cell lines that were transfected with either a vector expressing shRNA targeting *Rex-1* (psiRNA_Rex-1) or *Lamb1* (psiRNA_Lamb1). A) There was a reduction in *Rex-1* expression observed in CCB psiRNA-Rex-1 #14 cell line compared to the control (C= CCB cDNA). B) There was a reduction in *Lamb1* expression in all 7 psiRNA_Lamb1 cell lines compared to the control (C= CCB cDNA).

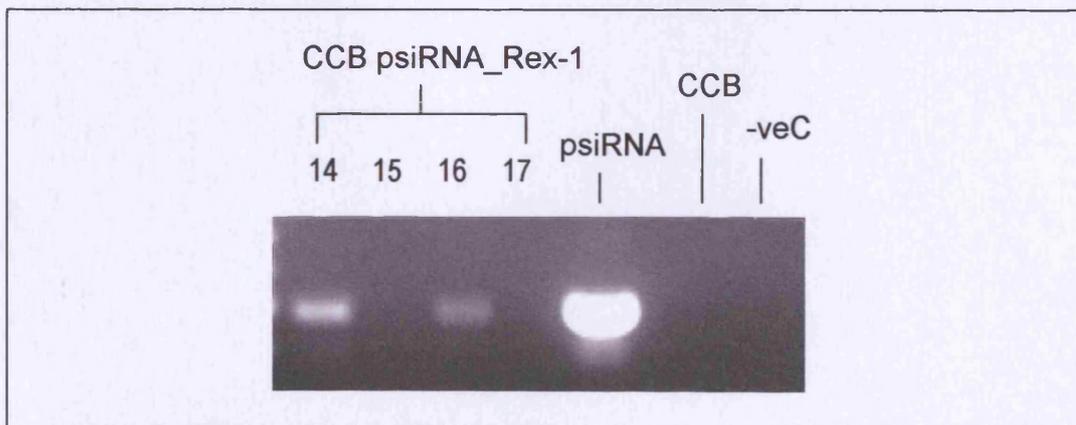


Figure 4.4. PCRs of gDNA taken from four psiRNA_Rex-1 CCB cell lines to look for integration of the psiRNA_Rex-1 vector. It can be seen that the plasmid is present in cell lines #14 and #16 but not in #15 and #17. PCR of psiRNA vector DNA was included as a positive control and gave a PCR product of the correct size. There were no bands in CCB gDNA PCR and PCR mix containing no DNA (-veC) as expected.

4.2.2. psi vector

The second vector used was the psi vector from Gerald Gish (Kunath *et al.*, 2003). A derivative of this vector (psiOct-4) was made (by Lars Grotewold, Edinburgh University, UK) expressing shRNA targeting *Oct-4* from the H1 RNA polymerase promoter (Figure 4.5). This vector was shown to induce differentiation of mES cells (personal communication).

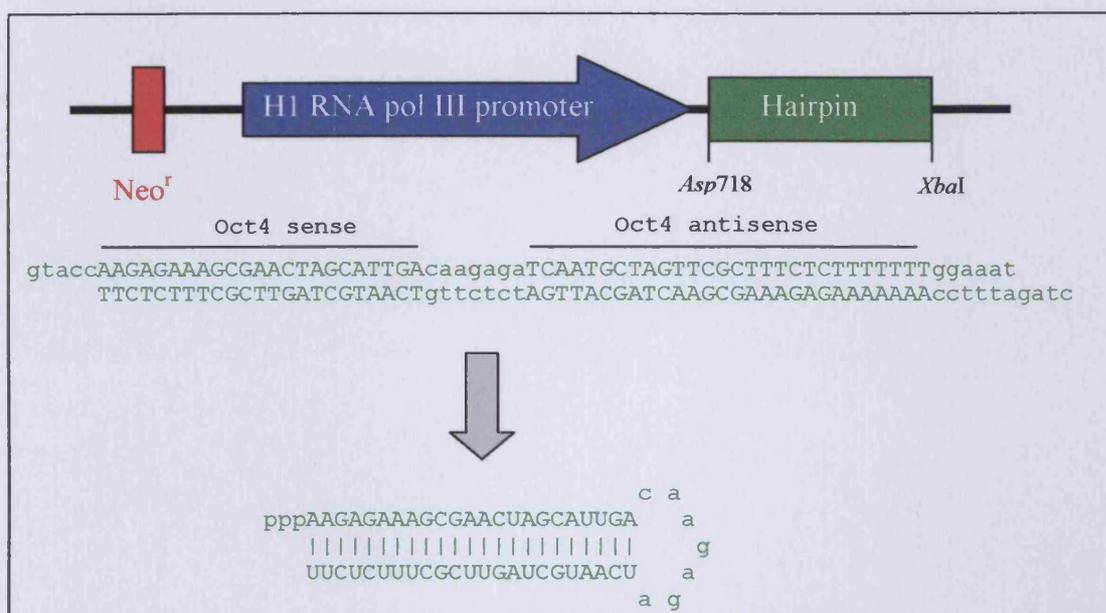


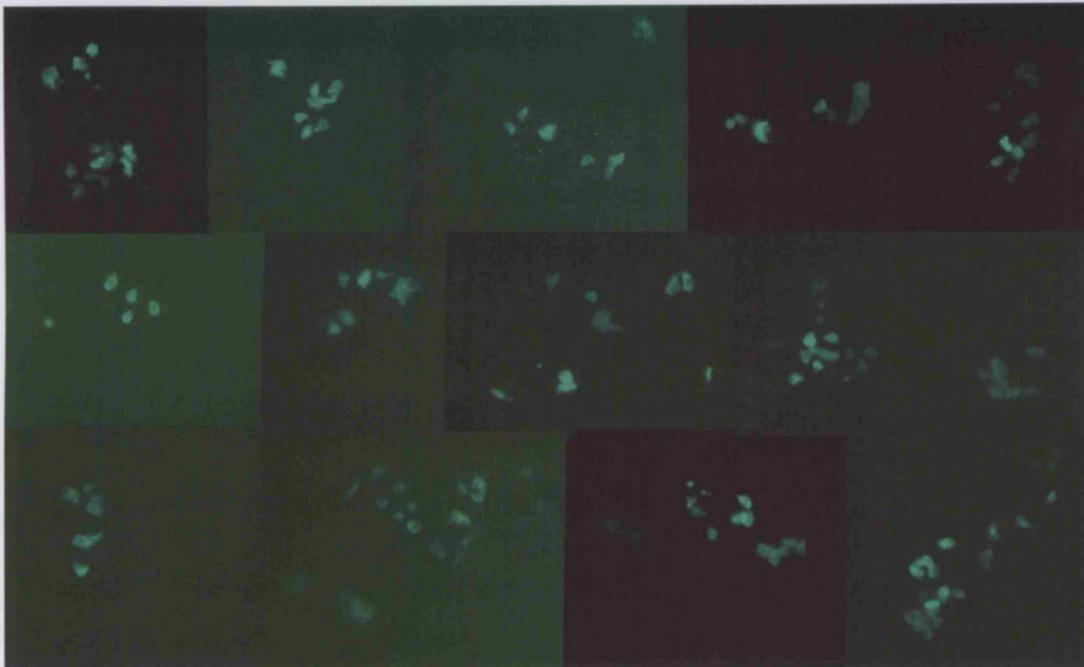
Figure 4.5. Main features of the psiOct-4 vector. *Oct-4* shRNA expression is under the control of the Human H1 RNA polymerase III promoter. The vector also has neomycin resistance, *Asp718* and *XbaI* sites for cloning of annealed oligos. Oligo for cloning had an inverted repeat of target sequence (22mer) separated by a 7bp loop sequence that when transcribes forms shRNA. [Adapted from (Kunath *et al.*, 2003).]

The aim was to use this vector to develop a system that could be used to study gene expression in mES cells involved in maintaining the self-renewal in mES cells whose knockdown would lead to differentiation of the cells resulting in a change in cells size. A reduction of *Oct-4* has been shown to induce differentiation of mES cells to trophoctoderm cells (Niwa *et al.*, 2000). This was observed by a change in cell morphology, as differentiated cells ‘flattened out’ over the culture surface and therefore were larger than mES cells expressing normal levels of *Oct-4*. Cotransfections using calcium phosphate (Section 2.2.2b) were performed of the psiOct-4 (Figure 4.5) and pTP6 vector (MmGFP expressing vector) (Appendix 1) into CCB mES cells (experimental cells) or psi vector without the *Oct-4* insert and pTP6

Chapter 4: RNA induced gene silencing using vectors expressing shRNAs

(control cells). The plasmids were transfected into cells at a ratio of 10:1, experimental cells with 6 μ g pTP6 and 600ng of psiOct-4 and control cells with 6 μ g pTP6 and 600ng psi. This ratio was used to ensure that all cells transfected with pTP6 would be transfected with the psiOct-4 or psi vector. Photographs were taken of transfected cells (control and experimental) identified as they express GFP (Figure 4.6). The size of the cells was measured using ImageJ (<http://rsb.info.nih.gov/ij/>).

Control cells



Experimental Cells

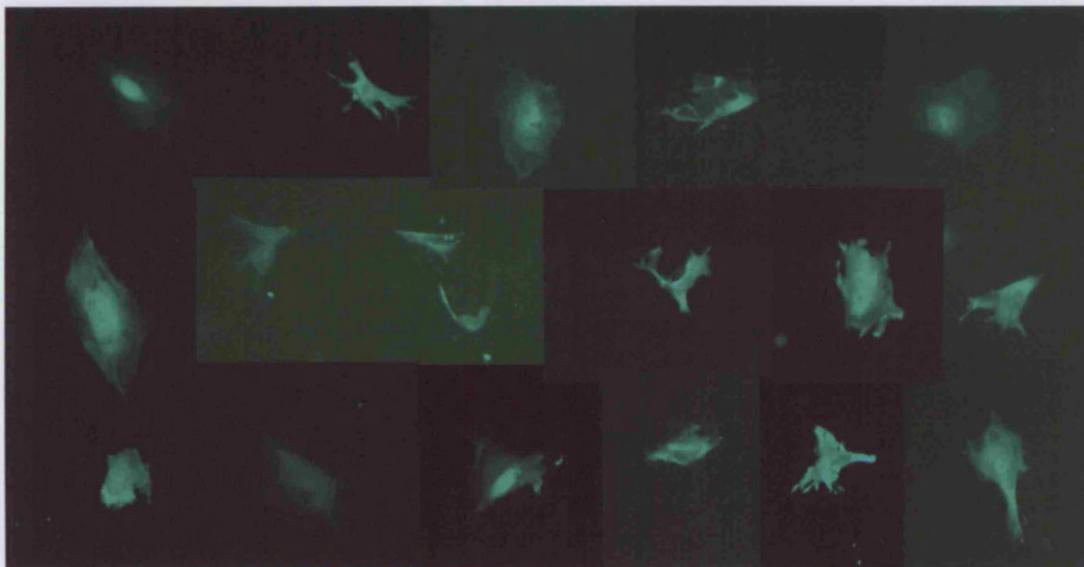


Figure 4.6. Morphology of CCB mES cells after *Oct-4* RNAi. Photographs of CCB mES cells taken 48h post transfection with a GFP expressing plasmid (pTP6) and psi (Control cells) or pTP6 and a vector expressing shRNA targeting *Oct-4* (Experimental cells). CCB mES Cells with *Oct-4* knockdown have differentiated and are larger than control cells.

The same experiment was repeated 3 times. It was observed that there was a difference in cell size between the experimental and control groups. To confirm the difference in cell size statistically, the number of cells measured to be equal to or less than $1000\mu\text{m}^2$ in area and the number of cells that were larger than $1000\mu\text{m}^2$ were compared using the chi-squared test. $1000\mu\text{m}^2$ was chosen as the cut off point as the majority of normal ES cells (control cells) fall within the range of 0 to $1000\mu\text{m}^2$. The value of chi-squared is calculated using the following equation:

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

O = observed value, E = expected value

Expected value for a cell = $\frac{\text{Row x Column total}}{\text{Grand total}}$

The expected and observed values for experiment 1 were calculated (Table 4.2).

Then the chi-squared value was calculated as follows:

$$\begin{aligned} \chi^2 &= \frac{(60-45)^2}{45} + \frac{(30-45)^2}{45} + \frac{(40-55)^2}{55} + \frac{(70-55)^2}{55} \\ &= 5 + 5 + 4.09 + 4.09 \\ &= 18.18 \end{aligned}$$

Values of chi-squared were determined in the same way for Experiments 2 and 3 (Table 4.3). The chi-squared values calculated were all larger than the p value at 0.001 (= 10.83) with 1 degree of freedom. Therefore it was concluded there is significant difference in cell size between experimental and control cells.

Cell Size	Number of control cells	Number of experimental cells	Total
< or = $1000\mu\text{m}^2$	60 45	30 45	90
> $1000\mu\text{m}^2$	40 55	70 55	110
Total	100	100	200

Table 4.2 Observed and expected values for Experiment 1

	Chi-squared value	Probability
Experiment 1	18.18	p<0.001
Experiment 2	93.08	p<0.001
Experiment 3	32.46	p<0.001

Table 4.3. Chi-squared values that show that the difference in cell size in experimental cells (transfected with psiOct-4 and pTP6) and control cells (psi and pTP6) is significant.

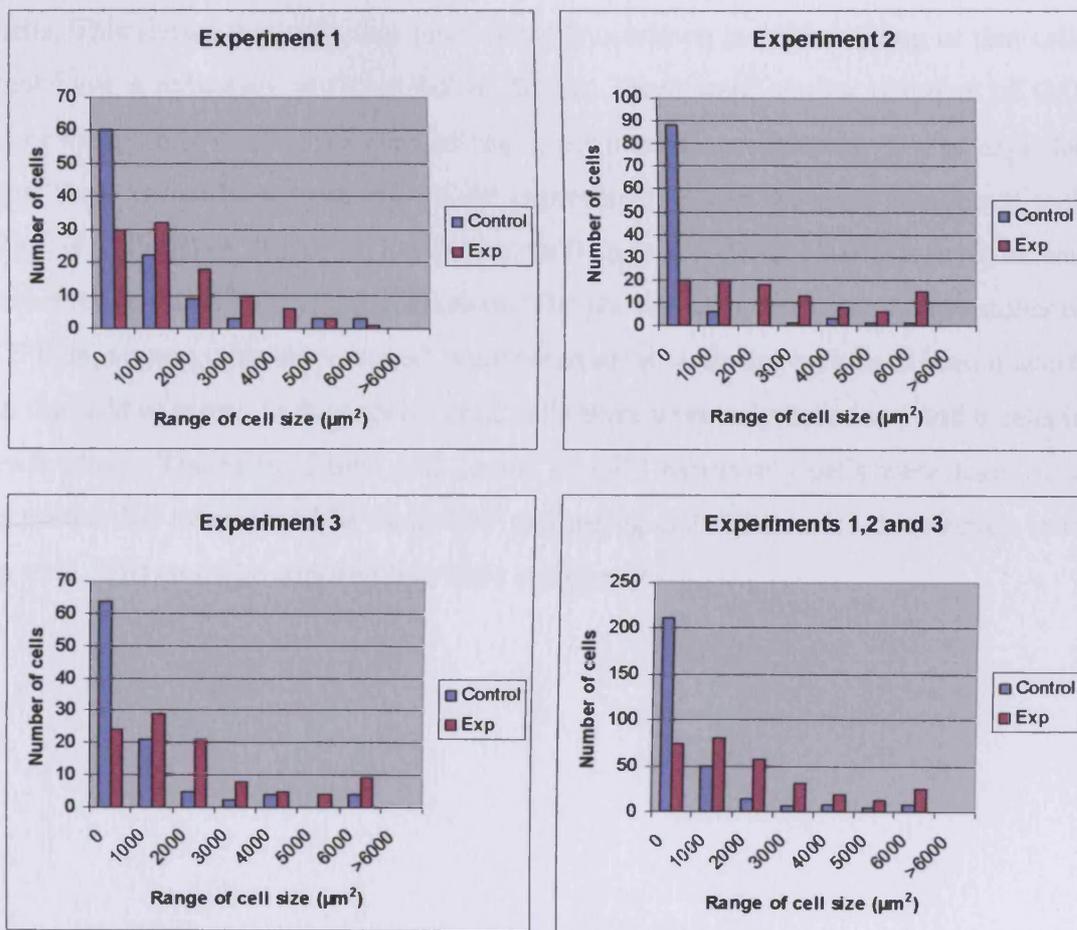


Figure 4.7. Change in CCB mES cell size 48h after *Oct-4* RNAi. Size of CCB mES cells were measured 48h after transfection with a vector expressing shRNA targeting *Oct-4* (Exp) or with the same vector without the shRNA (control). There was shown to be an increase in cell size upon knockdown of *Oct-4*.

Chapter 4: RNA induced gene silencing using vectors expressing shRNAs

The number of cells expressing GFP was very low in these experiments when transfected at a ratio of 1:10. In order to increase the number of cells seen to be transfected different ratios of psiOct-4/pTP6 vector were tried, 1:1 and 1:3 to compare to 1:10 ratio. This was to increase the number of cells transfected whilst ensuring that the cells analysed were transfected with both vectors. The experiment was conducted as previously described except for the amount of plasmid DNA used (Figure 4.9).

The number of cells expressing GFP was counted in 64 photos that were taken of each experiment and control transfection (Table 4.4). It can be seen that the number of GFP expressing cells was less at each ratio in the experimental compared to the control cells. This shows that cells that have *Oct-4* knockdown are either dying or that cells that have a reduction in *Oct-4* fail to divide. There were similar numbers of GFP expressing cells counted in each of the experimental transfections. It was expected that there would have been more GFP expressing cells in the cells transfected with 3µg of pTP6 DNA (Ratio 1:1) compared to 0.6µg of pTP6 DNA (Ratio 1:10) as was observed in the control cell transfections. The photographs, from which the number of GFP expressing cells was counted, were taken so as to always include at least one cell in the field of view. In the experimental cells there were only between 1 and 6 cells in each photo. Therefore if total cell counts of GFP expressing cells were taken it is expected that there would be more GFP expressing cells when a 1:1 transfection ratio is used. This could be confirmed by flow cytometry.

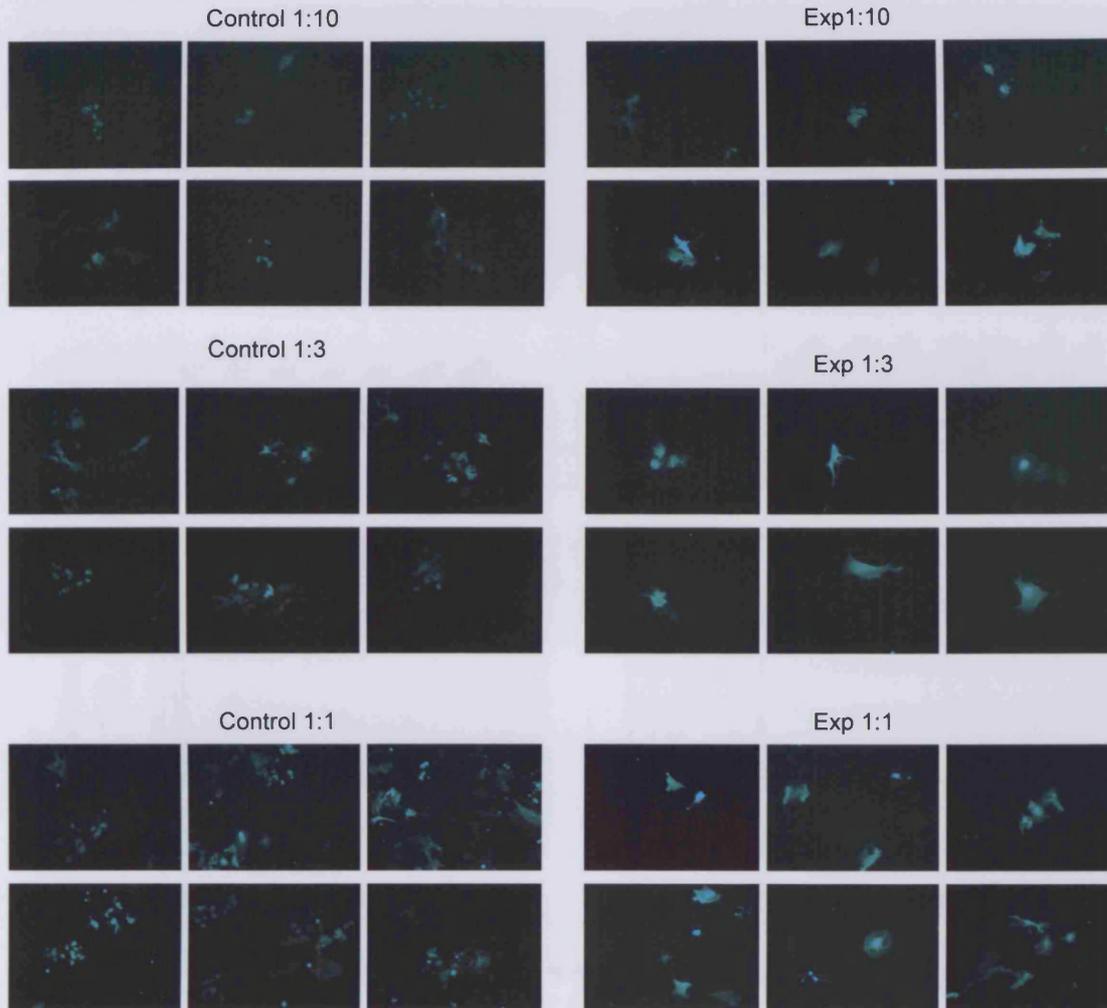


Figure 4.8. CCB mES cells with RNAi induced knockdown of *Oct-4*. Experimental cells were transfected with GFP expressing vector (pTP6) and vector expressing shRNA targeting *Oct-4* (psiOct-4) at the ratios indicated. Control cells were transfected with pTP6 and the psi vector that does not contain a shRNA. Photographs indicate that there are more GFP expressing cells in the controls compared to the experimental.

Ratio of pTP6:psi/psiOct-4	Number of GFP expressing cells	
	Control transfections	Experimental transfections
1:10	469	170
1:3	620	157
1:1	1417	188

Table 4.4. Number of GFP expressing CCB mES cells 48h after transfection. Experimental cells were transfected with GFP expressing vector (pTP6) and vector expressing shRNA targeting *Oct-4* (psiOct-4) at the ratios indicated. Control cells were transfected with pTP6 and psi vector not containing a shRNA. Photographs were taken such that GFP-expressing cells were in the field of view therefore as there are very few GFP expressing cells in cells transfected with psiOct-4 then similar number of cells were seen for all experimental studies.

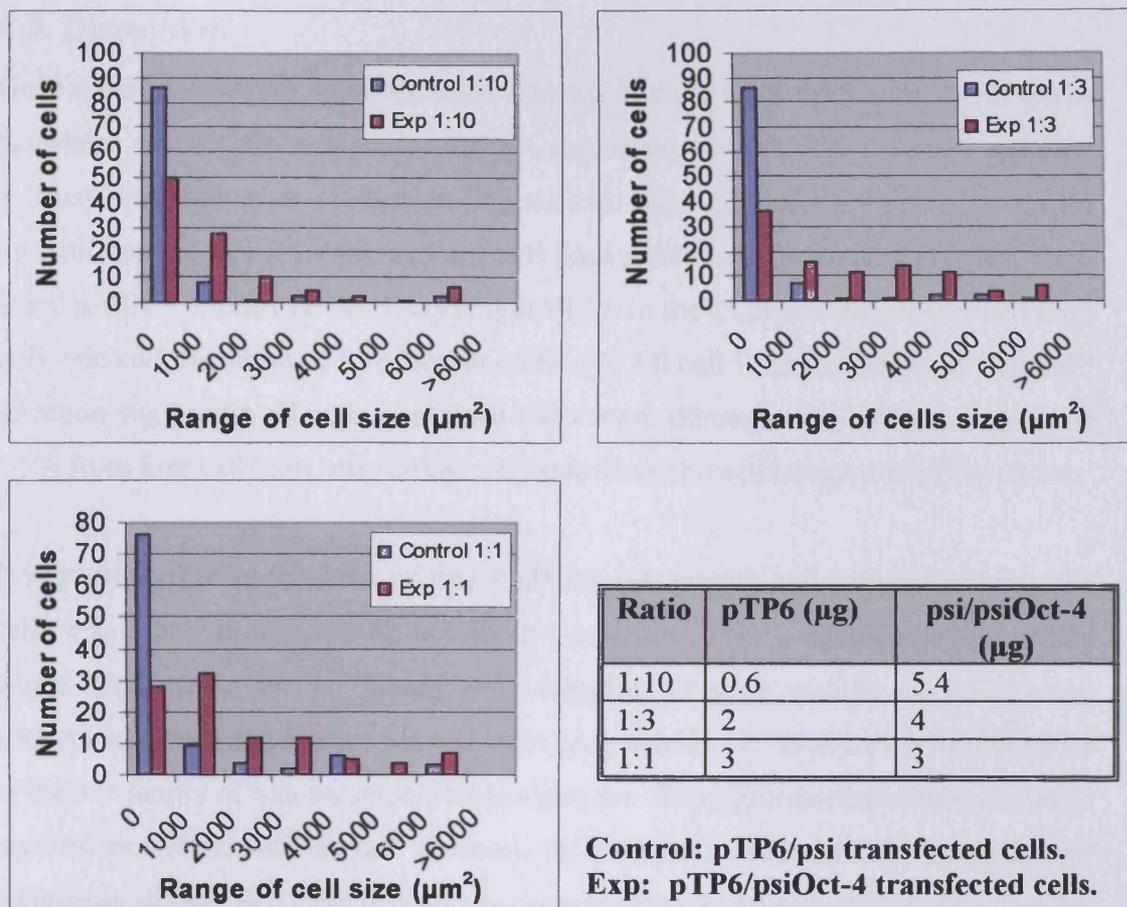


Figure 4.9. Change in CCB mES cell size 48h after *Oct-4* knockdown by RNAi. Different ratios of GFP expressing vector (pTP6) and Oct-4 shRNA expressing vector (psiOct-4) were transfected. This was to increase the number of cells that are available for analysis whilst still ensuring that cells were transfected with both plasmids.

Ratio of psiOct-4/psi:pTP6 Transfected	Chi-squared value	Probability
1:10	31.20	p<0.001
1:3	52.56	p<0.001
1:1	46.16	p<0.001

Table 4.5 Chi-squared values for CCB cells transfected with different amounts of pTP6 and psiOct-4/psi. Chi-squared values are all larger than the p value at 0.001

The chi-squared values were calculated and the resulting p values were all greater than the p value at 0.001 (= 10.38) with 1 degree of freedom (Table 4.5). Therefore it can be concluded there is significant difference in cell size between experimental and control cells in all of the experiments. Therefore in future a ratio of shRNA vector to GFP expressing vector of 1:1 will be used.

4.3. Discussion

Gene specific silencing has been demonstrated in these CCB mES cells. Three genes have been specifically targeted by shRNA expressing vectors, *Rex-1*, *Lamb1* and *Oct-4*. There was seen to be a reduction of gene expression of *Rex-1* and *Lamb1* as shown by semi-quantitative RT-PCR in some cell lines stably transfected with psiRNA_*Rex-1* and psiRNA_*Lamb1* respectively (Figure 4.3). In the CCB psiRNA_*Rex-1* cell lines only one cell line showed a reduction of *Rex-1*. All cell lines had survived Zeocin™ selection suggesting all cells contained the vector although when PCRs of genomic DNA from four cell lines was looked only two lines showed integration of the vector.

It is possible that knockdown of *Rex-1* affects cell growth and survival and that the cells with *Rex-1* knockdown do not survive selection. This is consistent with a study where the genes *cYes* and *Nanog* were targeted for gene specific silencing using siRNAs in mouse and human ES cell lines (Anneren *et al.*, 2004). *cYes* is a member of the Src family of non-receptor tyrosine kinases. These proteins have been shown to regulate processes such as cell division, differentiation and survival. Anneren and colleagues showed that *cYes* is highly expressed in mES and hES cells, is regulated by LIF and is downregulated when ES cells differentiate. In their experiments they were able to reduce the expression of *cYes* transiently but could not establish stable clones. ES cells were transfected with *cYes* shRNA vector and pTP6 (that expresses MmGFP and has puromycin resistance) and then cells were puromycin-selected. There were found to be less colonies in *cYes* shRNA transfected cells compared to control cells only transfected with the puromycin vector. Also the colonies that were present did not have lower *cYes* levels. Similar results were found when targeting *Nanog* for knockdown. Two reasons for this were suggested, either that cells had impaired growth and survival due to *Nanog/cYes* knockdown or that differentiation of the cells due to the knockdown of these genes made them more sensitive to puromycin.

In demonstrating the knockdown of *Rex-1* using my psiRNA-*Rex-1* plasmid targeting the 5'UTR of the gene of interest has been shown to be effective for shRNA induced gene silencing in these cells (Figure 4.3). Targeting the 5'UTR may result in the removal of the stabilising cap and degradation of the mRNA or inhibition of translation in a similar way to miRNAs (Section 1.1.1A). The 5'UTR of the hepatitis

Chapter 4: RNA induced gene silencing using vectors expressing shRNAs

C virus (HVC) genome was targeted for RNAi induced gene silencing resulting in inhibition of HVC replication (Yokota *et al.*, 2003).

Gene specific silencing of *Oct-4* was shown to induce a change in phenotype of the cells as has been shown in other studies in mES cells (Carpenter and Zernicka-Goetz, 2004; Takada *et al.*, 2005; Velkey and O'Shea, 2003). Knockdown of *Oct-4* was found to affect the growth of cells as there were a lot less GFP expressing cells in those transfected with *Oct-4* shRNA expressing vector compared to the control vector 48h post transfection (Table 4.4). In a study by Matin and colleagues a reduction in growth rate of cells was also observed after knockdown of *Oct-4* in hES cells (Matin *et al.*, 2004).

It has been shown that the psi vector used in this study can be used to generate transgenic mice when stably transfected into mES cells (Kunath *et al.*, 2003). The *Rasal* gene was targeted and it was found embryos derived from the shRNA expressing mES cells had the same phenotype as that seen in the *Rasal* null mutation that was previously described (Henkemeyer *et al.*, 1995).

These experiments have highlighted the limitations of shRNA based method for studying genes important in maintaining pluripotency of stem cells. A reduction in expression of these genes reduces cell growth and these cells are selected against. In the case of the psiOct-4 transfection experiment, as not all cells were transfected it is difficult to get a large number of cells for proper analysis. In order to study the function of *Oct-4*, *Nanog*, *cYes* and other genes involved in maintaining the pluripotency of mES *in vivo* an inducible system is necessary. In such a system mES cells would be transfected with the RNAi vector. Then cell lines containing the vector would be established before induction of gene silencing by switching on the expression of the hairpin dsRNA.

Chapter 5

Inducible expression of hairpin dsRNA for gene specific silencing

5. Inducible expression of hairpin dsRNA for gene specific silencing

5.1. Introduction

Inducible expression of hairpin dsRNA for gene specific silencing at specific time points would be advantageous especially when studying genes that affect cell viability. A cell line that contains an RNAi vector can be established before the targeted gene is silenced by 'switching on' expression of the hairpin RNA. *In vivo* inducible systems are particularly useful for studying embryonic lethal genes as their function can then be studied in adult animals.

One system that has been developed for efficiently regulating genes in eukaryotic cells utilises tetracycline controlled transactivators (Gossen and Bujard, 1995). The tetracycline responsive regulatory system (Tet system) consists of two components, the tetracycline-responsive transcriptional activator protein (tTA) in case of Tet-off system or the reverse tTA (rtTA) in the Tet-On system (Gossen *et al.*, 1995) and a gene expressed from a tetracycline regulated promoter (P_{tet}).

The tTA is made of a fusion protein between the repressor (TetR) of the *Tn10* tetracycline resistance operon of *E. coli* and a transcriptional activation domain of the VP16 protein of herpes simplex virus. rtTA is similar to tTA except that the TetR has four amino acids changes and is named rTetR. P_{tet} contains a minimal promoter (such as CMV that lacks the enhancer) and an array of tet operator (*tetO*) sequences called the tet-responsive element (TRE) (Strathdee *et al.*, 1999). In the 'Tet-Off' system the rTA fusion protein binds to the *tetO* sequences inducing expression of the gene under the CMV minimal promoter. Presence of tetracycline or doxycycline prevents binding of the rTA and therefore transcription is prevented.

In the 'Tet-On' system rtTA can only bind to the TRE in the presence of doxycycline and then activates transcription from the P_{CMV} minimal promoter (Figure 5.1). Therefore expression of the gene of interest is 'switched on' when doxycycline is added. The 'Tet-On' system was used in my study and the vector used, pBI-EGFP, contained a bi-directional promoter that has the TRE positioned between two identical

Chapter 5: Inducible expression of hairpin dsRNA for gene specific silencing

minimal CMV promoters (P_{minCMV}). One promoter controls the expression of green fluorescent protein (GFP) and the second promoter controls the expression of hairpin dsRNA that was cloned in. Therefore it was predicted that once cell lines had been established containing the two vectors addition of doxycycline would induce gene specific silencing and also expression of the reporter GFP (Figure 5.1).

It has been shown that hairpin dsRNA (hpRNA) induces specific gene silencing in undifferentiated ES cells (Yang *et al.*, 2001) as they do not exhibit an interferon response as seen with other mammalian cells. ES cells were transfected with dsRNA of 547bp targeting EGFP sequence made by *in vitro* expression or with a plasmid expressing the same dsRNA as a hairpin. There was found to be a specific and dose dependent reduction in EGFP expression whilst the expression of β -galactosidase did not change. This study demonstrated that transfection of dsRNA or hpRNA is suitable for RNAi induced gene silencing in ES cells. The use of dsRNA also overcomes the problem of only certain siRNAs inducing gene silencing as the dsRNA is cleaved by Dicer to produce 'pools' of siRNAs that target the gene in several places.

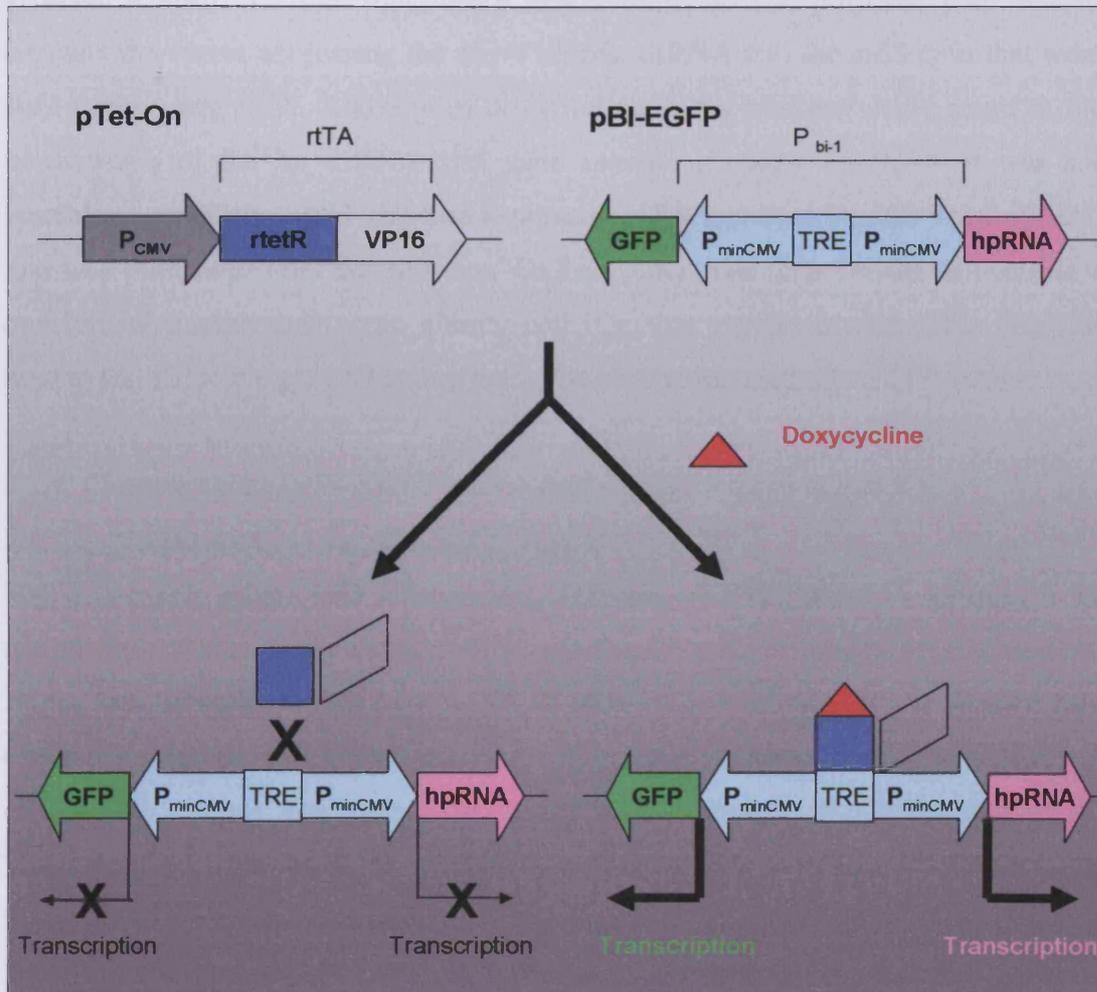


Figure 5.1. Tet-On system. The system consists of two components, the pTet-On vector containing a strong immediate early promoter of cytomegalovirus (P_{CMV}) from which the reverse tetracycline-responsive transcriptional activator protein, rtTA, is constitutively expressed. rtTA is made of a fusion protein between the repressor (rTetR) of the Tn10 tetracycline resistance operon of *E. coli* and a transcriptional activation domain of the VP16 protein of herpes simplex virus. The second vector contains a bidirectional promoter that has the tetracycline response element (TRE) positioned between two identical minimal CMV promoters (P_{minCMV}). One promoter controls the expression of green fluorescence protein (GFP) and the second promoter controls the expression of hairpin dsRNA (hpRNA) that had been cloned in. rtTA binds to the TRE when doxycycline is present and activates transcription of EGFP and the hairpin RNA (Gossen *et al.*, 1995).

5.2. Experimental Approach and Results

A vector expressing hairpin dsRNA from a tetracycline responsive bi-directional promoter was constructed for targeting *Oct-4*. During this time an attempt was made to make a mES cell line that stably expresses rtTA. The proposed plan was to transfect the vector expressing the *Oct-4* hairpin dsRNA into the mES cells that were stably expressing rtTA. Addition of doxycycline to the medium would result in the transcription of the hp dsRNA and gene specific silencing of *Oct-4*. It was not possible to establish a mES cell line expressing rtTA therefore the 293 Tet-On™ cell line was purchased (BD Biosciences, Oxford, UK). The 293 Tet-On cell line is a transformed human embryonic kidney cell line that expresses rtTA. This line was used to test inducible gene silencing using the aforementioned pBI-EGFP vector.

5.2.1. Construction of pBI-EGFP vector that expresses hairpin dsRNA

5.2.1.a. Modifications to the pBI-EGFP Vector

The pBI-EGFP vector (BD Biosciences, Oxford, UK) (Figure 5.1) contains a bi-directional promoter that expresses *GFP* and also the gene of interest once it has been cloned into the multiple cloning site (MCS) of the vector. In order to improve the ease of cloning inverted repeats into this vector the multiple cloning site of pBI-EGFP was modified to include new unique restriction sites including *SpeI*, *ClaI*, and *BstBI*. These sites were chosen as, together with *NheI* already present in the MCS, pairs of these enzymes produce compatible ends. Fragments digested with *SpeI* can ligate to DNA cut with either *SpeI* or *NheI*, likewise *BstBI* digested DNA can ligate to vectors cut with either *BstBI* or *ClaI* (Figure 5.7). There was already a *SpeI* site present in the pBI-EGFP vector (position 5103) that was not in an expressed region of the vector. Therefore this site was removed by digestion of the pBI-EGFP vector with *SpeI* restriction enzyme (Section 2.3.10b) and ligation of a linker sequence ('*SpeI* linker') that removes the site (Figure 5.2). The modified plasmid (pBI-EGFP*) was transformed into DH5α cells (Section 2.3.15) and colonies were picked and cultured in LB broth containing ampicillin. Plasmid DNA was prepared from the cultures (Section 2.3.6) and absence of the *SpeI* site was confirmed by *SpeI* restriction digest.

In order to clone the new restriction sites into the MCS of pBI-EGFP* a pair of oligos were designed that when annealed (Section 2.3.9) contained *SpeI*, *ClaI*, and *BstBI* sites (respectively) (Table 2.11) and also had 'overhanging' *MluI* ends. This allowed ligation, using T4 DNA ligase (Section 2.3.13), of the duplex into the *MluI* site present in the MCS of pBI-EGFP*. The new vector named pBI-EGFP*_MCS was transformed into DH5 α cells (Section 2.3.15) and cultured on ampicillin LB agar plates. Colonies were picked and used in a PCR screen to determine the presence of the MCS insert (Section 2.3.16) using pBI-MCS primers (Table 2.7). Plasmids containing the insert can be identified by having a larger PCR product than the plasmids with no insert. In this case the difference was only 52bp so PCR products were run on a higher percentage agarose gel (2%) as this allows better separation of smaller PCR products (Figure 5.3).

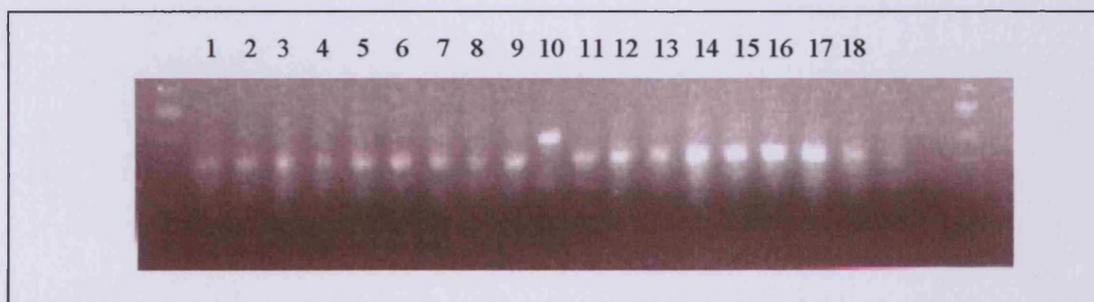


Figure 5.3. Results of PCR Screen for presence of new MCS. PCR screen was performed on plasmid DNA extracted from eighteen different bacterial colonies to look for plasmids containing the insert. The presence of larger sized PCR product in #10 shows that the insert has ligated into this plasmid. This plasmid was named pBI-EGFP*MCS.

The plasmids that were found to contain the insert were confirmed by restriction digests (Figure 5.4). It was seen that the pBI-EGFP*MCS #10 plasmid was digested by *SpeI*, *NheI*, and *BstBI* but not with *ClaI*. In subsequent PCR screens several other plasmids were identified as containing the insert and were also found to linearise with *SpeI*, *NheI*, and *BstBI* but not *ClaI*. The plasmids were sequenced (Section 2.3.17) and found to contain the insert with a *ClaI* site of the correct sequence. After investigating reasons why the plasmid was not linearised by the *ClaI* restriction enzyme it was discovered that this site can be methylated. Dam methylase is present in most *E. coli* including the DH5 α strain that was used. This enzyme methylates the adenine

Chapter 5: Inducible expression of hairpin dsRNA for gene specific silencing

residues in the sequence GATC. The sequence of the *Cla*I site is ATCGAT but the insert was also followed by a C. Therefore the *Cla*I site was protected from cleavage due to the presence of the methylated A base. Transformation of the pBI-EGFP*MCS plasmid into *Dam*⁻ *E.coli*, GM2163 strain (Table 2.5) meant there was no methylation within the *Cla*I restriction site and two plasmids were selected that were linearised once with *Bst*BI, *Cla*I, *Mlu*I, *Spe*I and *Xho*I as expected (Figure 5.5). The plasmid inserts were sequenced and one of the plasmids was shown to contain the insert in the correct orientation (pBI-EGFP*MCS#3). The plasmid was ready for the cloning in of inverted repeated DNA sequences of genes of interest that when expressed would produce hairpin RNA.

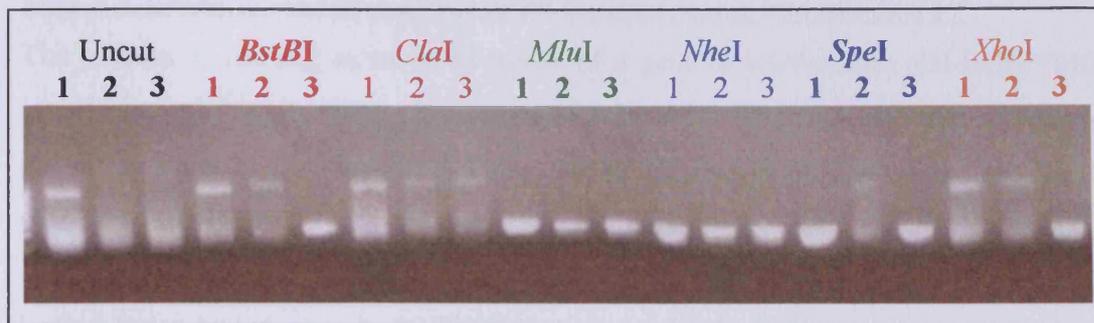


Figure 5.4. Restriction digest products of pBI-EGFP* MCS that had been transformed into DH5a *E. coli*. After ligation of the new MCS into the pBI-EGFP vector the presence of the insert was confirmed by a number of restriction digests. 1 and 2 were control vectors (pBI-EGFP original vector, and pBI-EGFP*) 3 was the test vector pBI-EGFP*MCS #10 from the PCR screen (Figure 5.3). The test vector was digested once (shown by the presence of a single band) with *BstBI*, *MluI*, *SpeI* and *XhoI*. *ClaI* should also have linearised the test vector.

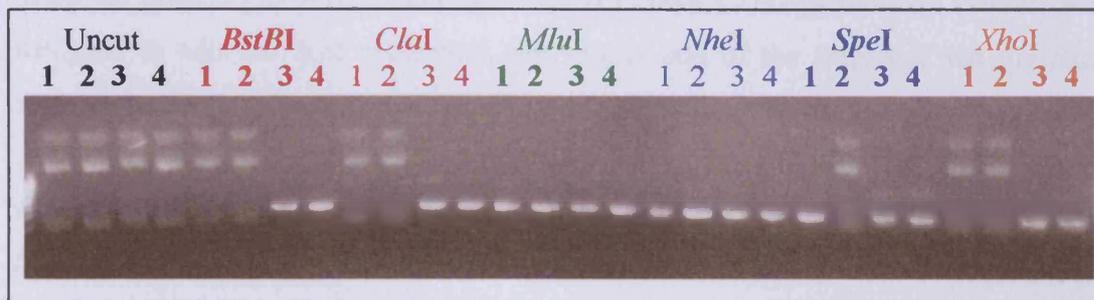


Figure 5.5. Restriction digest products of pBI-EGFP*MCS vectors that had been transformed into Dam⁻ *E. coli*. The presence of the insert in the pBI-EGFP*MCS (3 and 4) vectors were tested for by restriction digests. Control vectors were also digested. 1 is the original pBI-EGFP vector, 2 is the modified pBI-EGFP* vector without the *SpeI* site. Both vectors (3 and 4) were shown to contain the insert as they were linearised with *BstBI*, *ClaI*, *MluI*, *SpeI* and *XhoI* as expected.

```
>pBI-EGFP*MCS#3
AGTCAGCTGACGCGTCGGGACTAGTCCCGATCGATGGACCATTGCAC
TCGAGCGGACTTTCGAAGCATGCGCGTGCTAGCGGCCCTAA

>pBI-EGFP*MCS#4
AGTCAGCTGACGCGCATGCTTTCGAAGTCCGCTCGAGTGCAATGGTCC
ATCGATCGGGACTAGTCCCGACGCGTGCTAGCGCGA
```

Figure 5.6. Sequencing of pBI-EGFP*MCS Vectors. Restriction sites into which inverted repeat sequences will be clones are highlighted as follows **TTCGAA** = *BstBI* site, **ATCGAT** = *ClaI* site, **ACTAGT** = *SpeI* site, **GCTAGC** = *NheI* site. The insert in pBI-EGFP*MCS#3 is of the correct sequence and orientation and therefore was used for the next stage of cloning. In pBI-EGFP*MCS#4 the insert has been ligated in the wrong way round.

5.2.1.b. Cloning of the *Oct-4* cDNA as an inverted repeat into pBI-EGFP*

The process of cloning an inverted repeat of a gene of interest into pBI-EGFP*MCS involved amplifying a region from its cDNA by PCR. The sequence that was chosen did not contain *BstBI*, *NheI* or *SpeI* restriction sites as these sites were used for the cloning of the insert. Primers that amplify the region were designed to add restriction enzyme sites (*SpeI* and *BstBI*) to the ends of the PCR product. This same fragment is ligated twice into digested pBI-EGFP*MCS. In the first instance it was cloned into the *SpeI* and *Clal* sites of the vector then, once the presence of this insert has been confirmed, into the *BstBI* and *NheI* sites of pBI-EGFP*MCS (Figure 5.7).

The first vector that was constructed was designed to knockdown *Oct-4*. A section of *Oct-4* was amplified by PCR from the pCMV-*Oct-4* vector (Scholer *et al.*, 1990) using the primers *Oct-4* IR_F and *Oct-4* IR_R (Table 2.7) (Appendix 2). Primers were designed to add the *SpeI* restriction site to one end of the fragment and the *BstBI* restriction site to the other end. The *Oct-4* PCR fragment was gel purified (Section 2.3.11) and digested first with *SpeI* and then with *BstBI* and then the *Oct-4* fragment was gel purified again. pBI-EGFP*MCS#3 was digested with *SpeI* first then *Clal* (Section 2.3.10b) Ligations were set up between the digested *Oct-4* fragment and digested pBI-EGFP*MCS#3 (Section 2.3.13) and then transformed into GM2163 strain of *E. coli* (Section 2.3.15). A PCR screen (Section 2.3.16) was performed of transformed colonies that were picked from plates to identify those containing the insert (Figure 5.8).

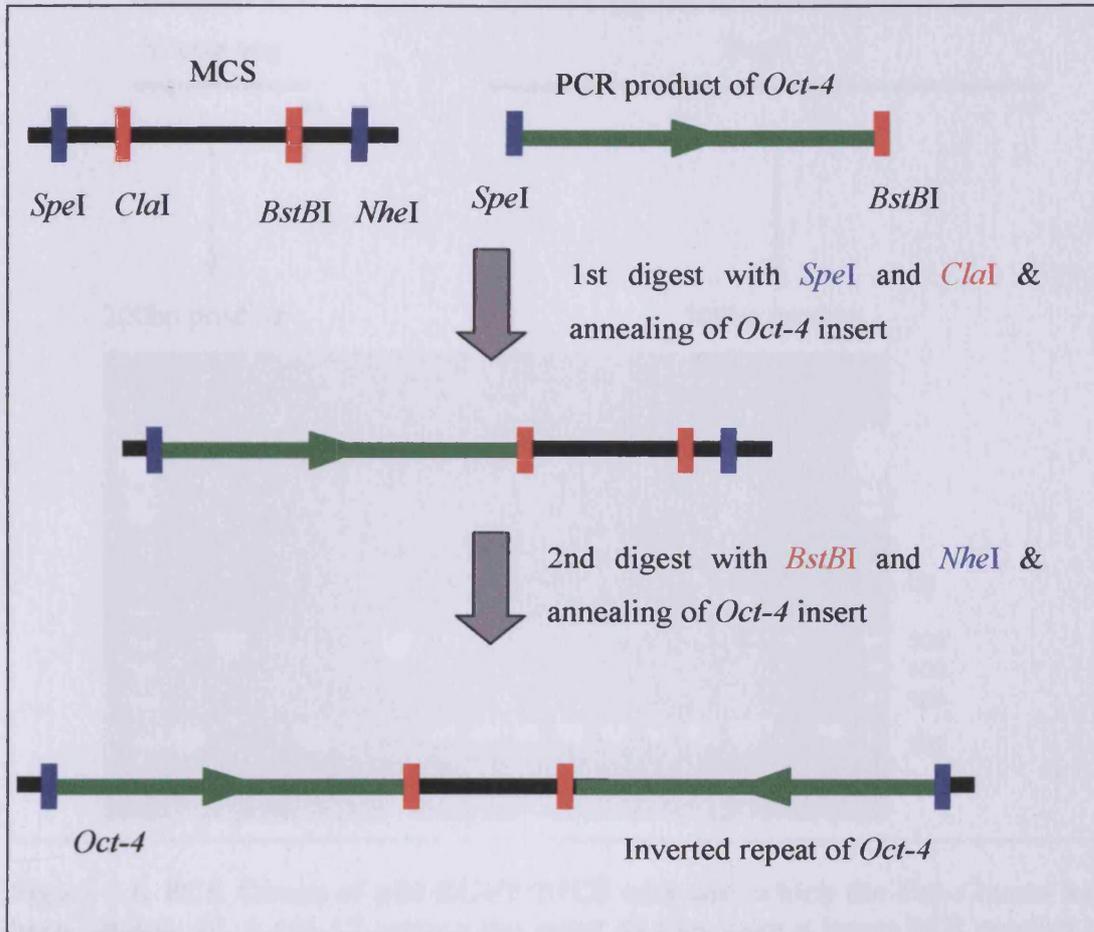


Figure 5.7. Cloning of inverted repeated into the multiple cloning site of pBI-EGFP*MCS. *SpeI* and *BstBI* restriction sites were added to the end of fragment of the gene of interest (*Oct-4*) amplified by PCR. pBI-EGFP*MCS vector was digested with *SpeI* and *ClaI* and *Oct-4* insert ligated into the vector in the 'forward' direction. Then vector was digested with *BstBI* and *NheI* and *Oct-4* ligated in opposite direction. The inverted repeat of *Oct-4* fragment forms hairpin RNA when it is transcribed.

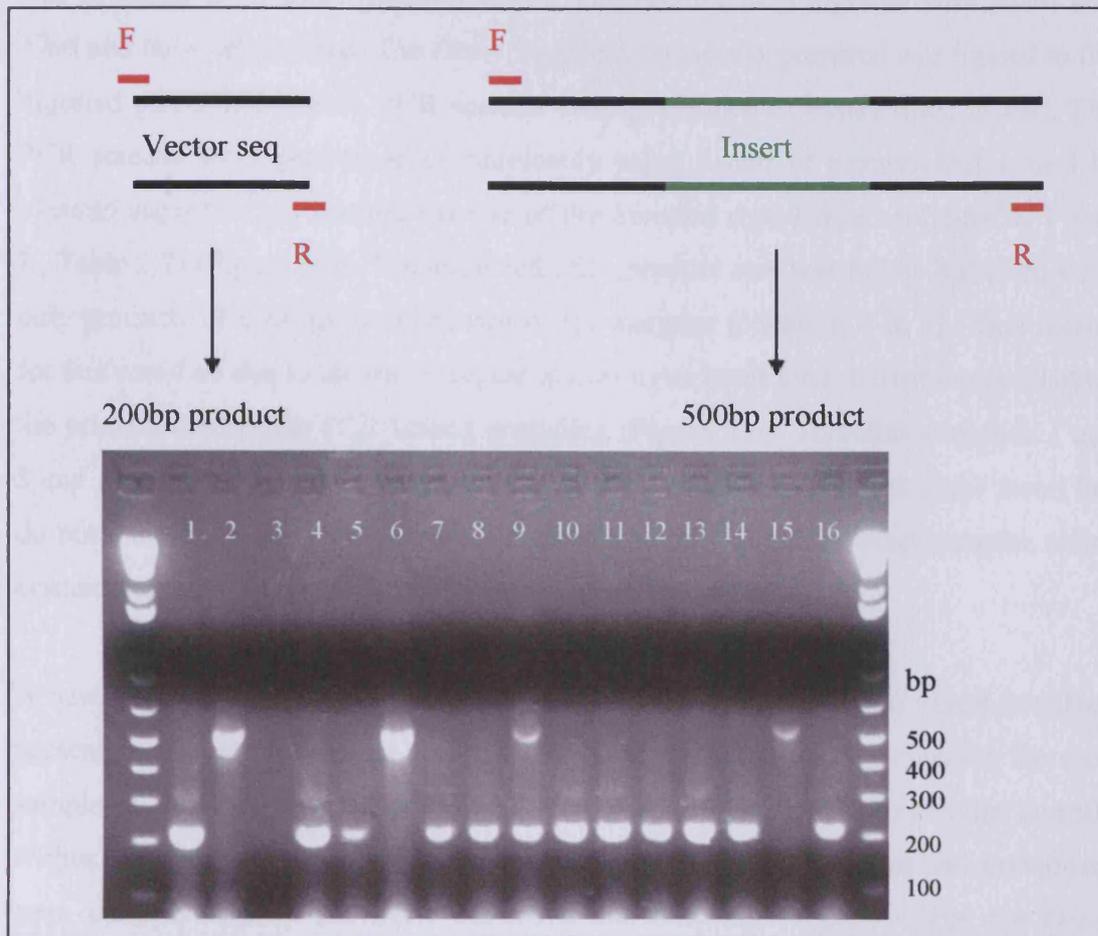


Figure 5.8. PCR Screen of pBI-EGFP*MCS with into which the *Oct-4* insert had been ligated. #2, 6 and 15 contain the insert as they gave a larger PCR product of 500bp.

Chapter 5: Inducible expression of hairpin dsRNA for gene specific silencing

Cultures with positive colonies (pBI-EGFP*Oct-4) were inoculated and plasmid DNA was prepared from them (Section 2.3.6). The plasmid was digested with *Bst*BI and *Nhe*I and then gel purified. The *Oct-4* fragment previously prepared was ligated to the digested pBI-EGFP*Oct-4. PCR screens were performed of transformed *E. coli*. The PCR screens were performed as previously using a pair of primers that anneal to plasmid sequence just outside the site of the inverted repeat insert (pBI-MCS_F and R, Table 2.7) (Figure 5.9). The expected PCR product size was 800bp but there were only products of c500bp found in two of the samples (plasmids 1 & 3). One reason for this could be due to the invert repeat annealing to itself after denaturing preventing the primers used in the PCR screen annealing (Figure 5.9). Therefore plasmids 1 and 3 and gave a PCR product corresponding to the presence of the first *Oct-4* insert but do not contain the inverted repeat. It is predicted that all of the other samples either contain the inverted repeat or have lost the insert altogether.

A new PCR screen was designed to investigate whether the inverted repeat is indeed present in all samples except 1 & 3. Two PCR reactions were performed for each sample. PCR #1 used primer pBI-MCS_F from the previous PCR screen that anneals within the plasmid sequence and a reverse primer, Oct-4 IR_R, that had previously been used to amplify the *Oct-4* sequence and therefore anneals within the *Oct-4* sequence insert (Figure 5.10). This PCR gives a product of c400bp that should be present in all of the samples. PCR #2 uses the pBI-MCS_R primer that anneals within the vector sequence and the Oct-4 IR_F primer that was used to amplify the *Oct-4* sequence. A PCR product of c400bp should be present, in PCR #2, when the inverted *Oct-4* sequence was present. This was observed in all plasmids except #1 and #3 therefore these vectors, unlike the others, do not contain the second *Oct-4* insert as had been predicted from my original PCR screen.

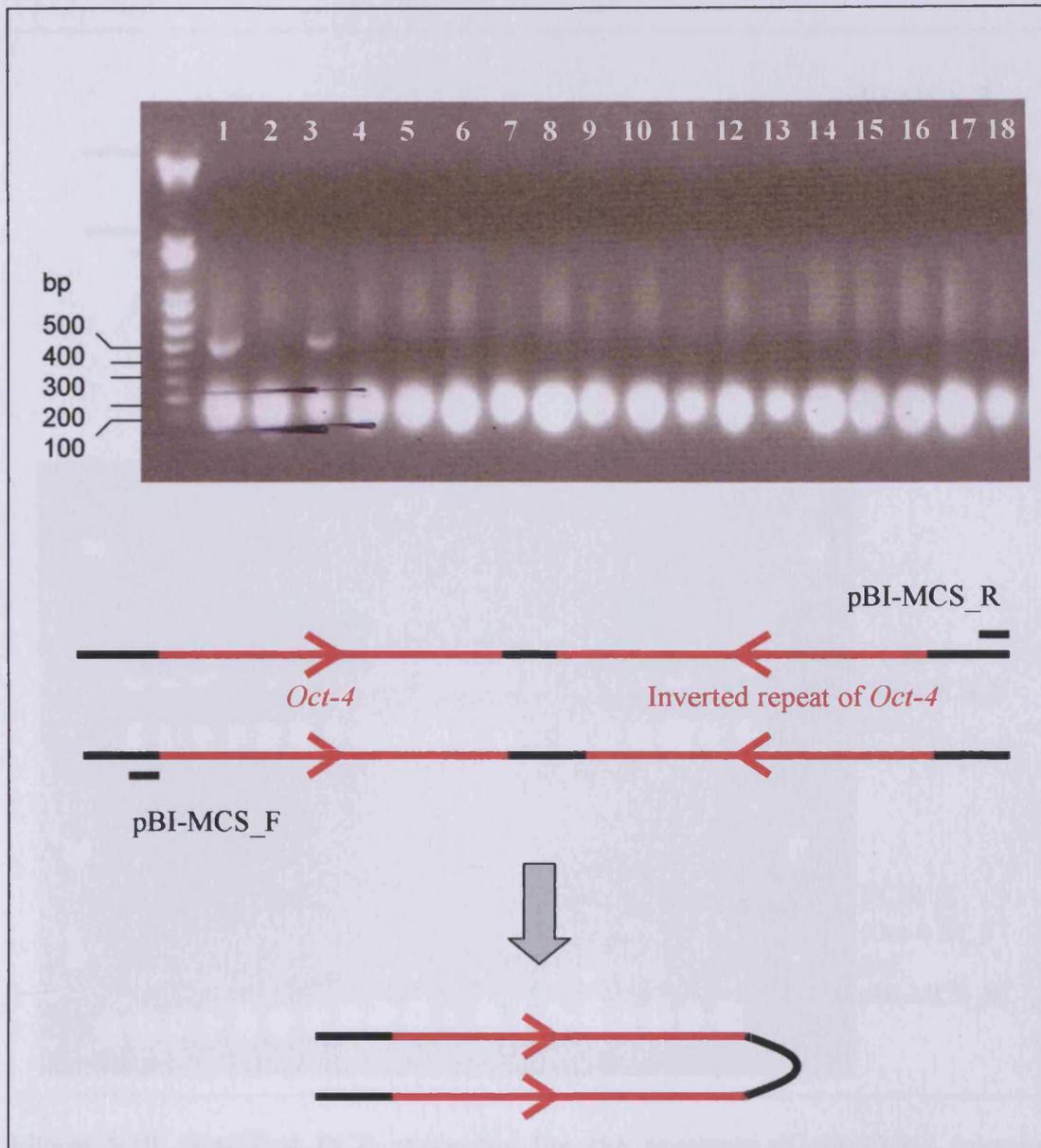


Figure 5.9. PCR screen of pBI-EGFP*Oct-4IR plasmids. It was expected that there would be a PCR product c800bp using primers pBI-MCS_F and pBI-MCS_R that anneal within the vector sequence just outside the site of cloning. There was no product of this size seen but there was a product present in two of the samples (1 & 3) at c500bp. One reason for this could be due to the invert repeat annealing to itself after denaturing before the primers can anneal. Therefore it is predicted that plasmids 1 and 3 and gave a PCR product corresponding to the present of first *Oct-4* insert but do not contain the inverted repeat. It is predicted that all of the other samples either contain the inverted repeat or have lost the insert.

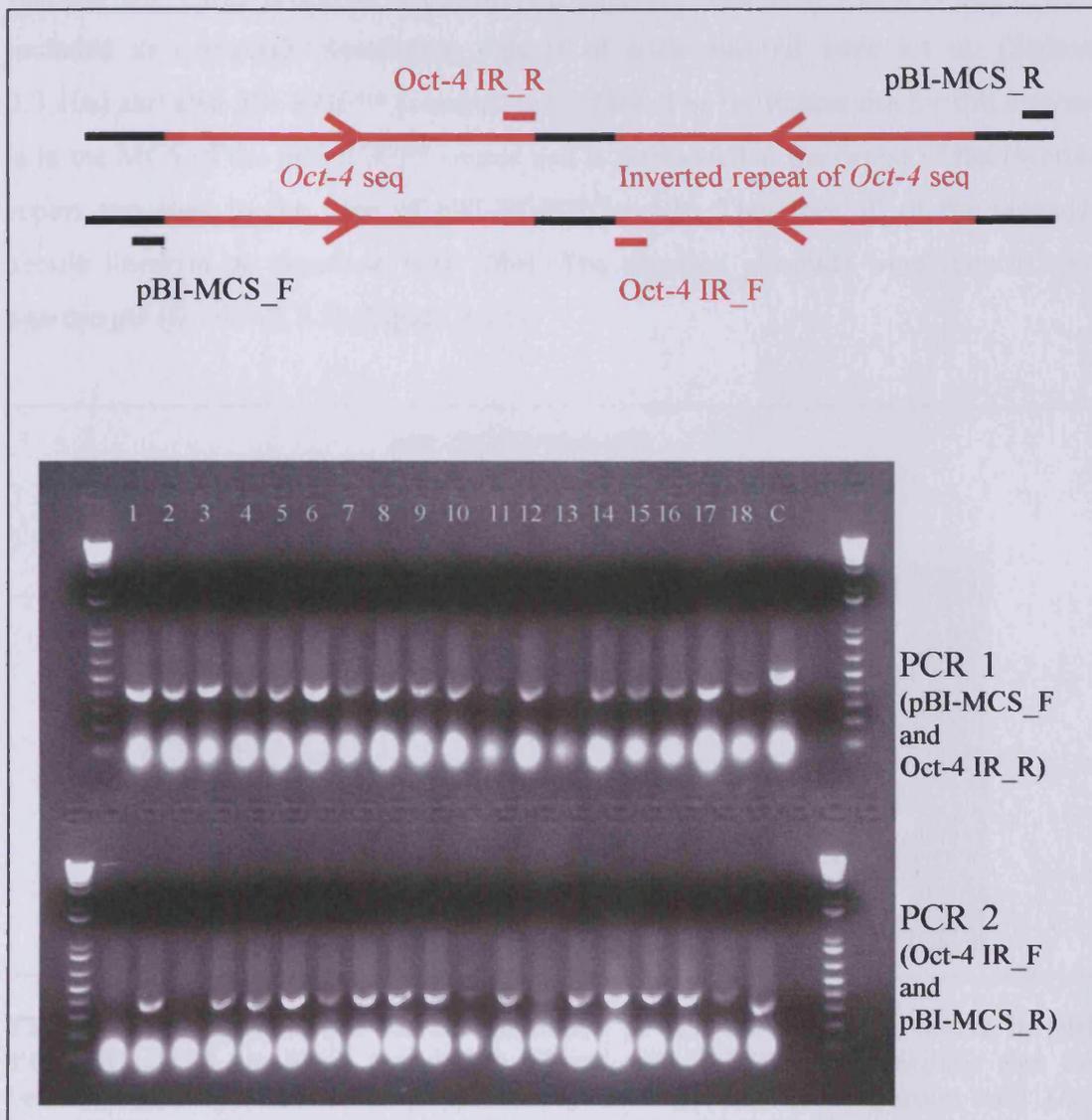


Figure 5.10. Modified PCR screening for the presence of the Oct-4 inverted repeat. Diagram shows the sites of annealing of the primers used for two PCR reactions designed to detect the presence of the inverted repeat. Red lines represent the *Oct-4* sequence and black lines the plasmid sequence. All plasmids analysed should have a PCR product of c400bp in PCR#1 as the parent plasmid contains the first *Oct-4* sequence. Presence of a PCR product in PCR #2 shows that the second *Oct-4* fragment has also been cloned in. Only plasmids 1 and 3 did not contain the inverted repeat as had been predicted from my original PCR screen. The control used was the original pBI-EGFP*Oct-4 plasmid and gave a slightly bigger product in PCR 1 as it had not been digested for a second time.

Plasmid DNA was prepared of plasmids (pBI-EGFP*Oct-4IR) 1 to 6 (1 and 3 were included as controls). Restriction digests of each plasmid were set up (Section 2.3.10a) and also pBI-EGFP* (control) with *Xho*I. The restriction site for this enzyme is in the MCS of the pBI-EGFP* vector and is positioned at the centre of the inverted repeat sequence in the case of pBI-EGFP*Oct-4IR. Therefore all of the plasmids should linearise on digestion with *Xho*I. The digested plasmids were run on 0.8% agarose gel (Section 2.3.1) (Figure 5.11).

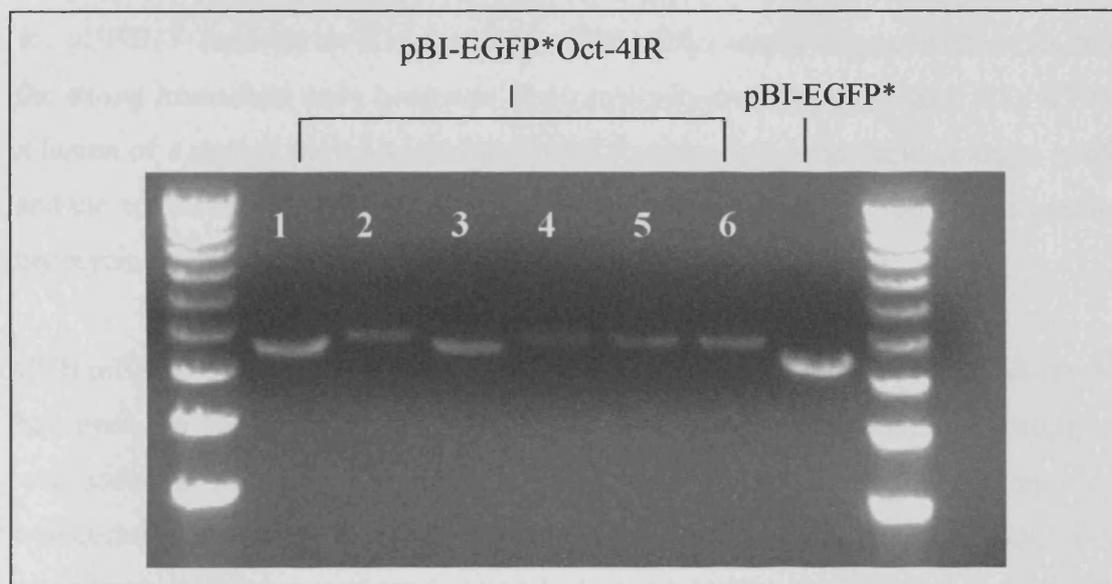


Figure 5.11. Confirmation of the presence of the second *Oct-4* insert in pBI-EGFP*Oct-4IR by *Xho*I restriction digest. pBI-EGFP*Oct-4 plasmids that had been analysed by PCR screen (Figure 5.10) were linearised by digestion with *Xho*I. pBI-EGFP that does not contain either *Oct-4* insert was included as a reference. The results were as expected. pBI-EGFP*Oct-4 1 and 3 were smaller than the rest of the pBI-EGFP*Oct-4IR vectors but larger than pBI-EGFP*, as shown by the distance migrated on the gel. Therefore this shows that 2, 4, 5, 6 contain the *Oct-4* inverted repeat.

Two sequencing reactions were set up for each pBI-EGFP*Oct-4IR plasmid (1-6) using either primers pBI-MCS_F or pBI-MCS_R (Table 2.7) using Big Dye v3.1™ (Web Scientific Ltd., Crewe, UK) (Section 2.3.17). Sequencing results were only obtained for plasmids 1 and 3, the plasmids not containing the *Oct-4* inverted repeat. It was predicted that the inverted repeat present in the other plasmids anneals to itself preventing successful sequencing. Therefore a tetracycline inducible vector was made that when transfected into cells should express both *EGFP* and hairpin dsRNA targeting *Oct-4* in the presence of doxycycline and rtTA. This vector was not able to

be tested in cells as a mES Tet-On cell line was not available and attempts to make one were unsuccessful (Section 5.2.2).

5.2.2. Construction of an ES cell line expressing *rtTA*

During the development of methods for cloning inverted repeat sequences into pBI-EGFP, attempts were made to make a mES cell line that stably expresses the reverse tetracycline-responsive transcriptional activator protein, *rtTA*. The vector used was the pTet-On (BD Biosciences, Oxford, UK) (Appendix 1) and was originally called the pUHD17-1neo vector (Gossen *et al.*, 1995). This vector expresses the *rtTA* from the strong immediate early promoter of cytomegalovirus (P_{CMV}) (Figure 5.1). *rtTA* is a fusion of a part of the tet repressor (TetR) that has four amino acid changes (*rtetR*) and the activation domain of the herpes simplex virus (HSV). The vector also contains neomycin resistance for selection of cells containing the vector.

CCB mES cells were electroporated (Section 2.2.2c) with *ScaI* linearised pTet-On that had been gel purified (Section 2.3.11) 48h after transfection neomycin (200 μ g/ml) was added to the culture medium for selection of the cells that had been stably transfected with pTet-On. 13 days after selection 48 colonies (CCB Tet-On) were picked and seeded into a 96 well plate (Section 2.2.4). These cell lines were expanded and the seeded into 24-well plates to test for the expression of *rtTA* from pTet-On by transfecting cells with pBI-EGFP vector and culturing with and without doxycycline. It was expected that cell lines expressing *rtTA* would express *GFP* from the pBI promoter when doxycycline was added to the culture medium. However, this was not observed in the 21 cell lines tested. Genomic DNA (gDNA) was extracted from the CCB Tet-On cell lines for analysis of integration of the Tet-On vector (Figure 5.12) using *rtTA_F* and R primers (Table 2.7) that anneal with the expressed region of the vector.

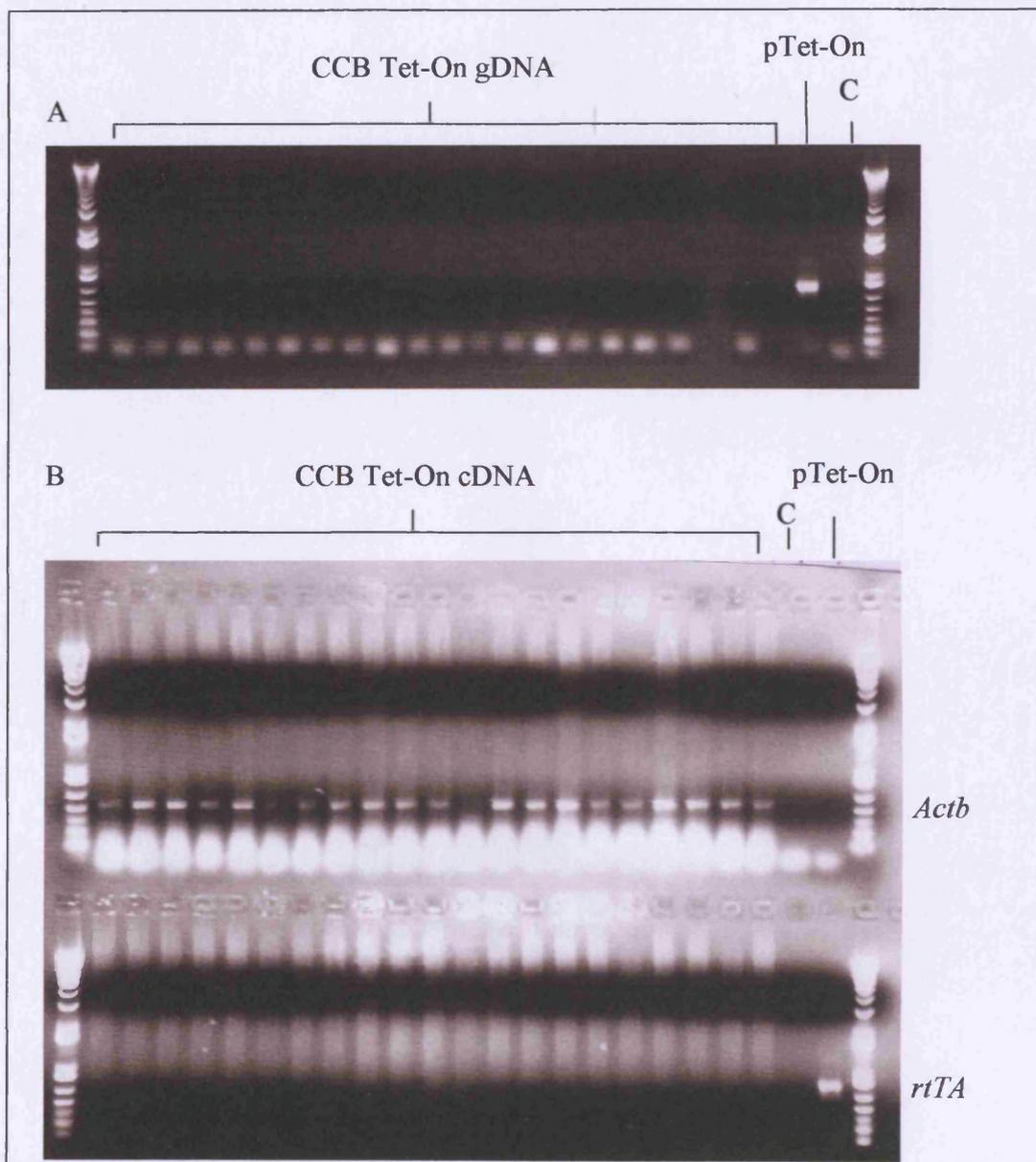


Figure 5.12. Analysis of CCB Tet-On cell lines A) PCRs of gDNA extracted from CCB Tet-On cell lines were performed using primers that anneal within the pTet-On thereby confirming whether or not the vector has integrated. A PCR product was only seen in the plasmid only control. B) PCRs were also performed on cDNA reverse transcribed from RNA extracted from the same CCB Tet-On cell lines. Expression of *Actb* was seen in most cell lines whereas *rITA* was not seen in any of the cell lines. The presence of a PCR product in the reaction set up with pTet-On vector confirms these PCR worked. (C is PCR mix only).

5.2.3. Construction and transfection of the pBI-EGFP expressing hpRNA targeting LaminA/C in 293 Tet-On cells

Due to the difficulty in constructing a pTet-On mES cell line the 293 Tet-On cell line was purchased (Clontech, Oxford, UK). The 293 Tet-On cell line is a transformed kidney cell line that expresses the reverse-tetracycline-controlled transactivator (rtTA). This cell line was used so that the system for inducible targeting of gene expression using the pBI-EGFP vector could be tested. The pBI-EGFP_Oct-4 vector could not be used in these cells as they are a human cell line. The target gene chosen was Lamin A/C (*LMNA*) as this gene had been targeted before for gene silencing (Elbashir et al., 2001a; Koper-Emde et al., 2004). A region of *LMNA* (155bp, Appendix 2) was amplified from cDNA that had been reverse-transcribed from RNA extracted from the 293 Tet-On cells (Section 2.3.4) using primers LMNA IR_F and R (Section 2.3.5) (Table 2.7).

The *LMNA* insert was cloned into the pBI-EGFP* vector as described for the Oct-4 fragment (Section 5.2.1). The vector that contained the inverted repeat for *LMNA* was named pBI-EGFP**LMNAIR*. The vector was prepared for transfection into cells by linearising it with *AseI* restriction enzyme (Section 2.3.10b). PGKHyg that expresses the hygromycin resistance gene from the mouse phosphoglycerate kinase (*pk1*) promoter was digested from the vector plox2PGKHyg using *AseI*. The linearised plasmids were gel purified (Section 2.3.11) then purified by ethanol precipitation (Section 2.3.12).

Then the digested and purified pBI-EGFP**LMNAIR* and PGKHyg fragments were transfected into HEK 293 Tet-On cells using calcium phosphate transfection (Section 2.2.2b). Control transfections were also performed: PGKHyg and the pBI-EGFP vector, pGKHyg vector only and pTP6 (GFP expressing plasmid) that confirmed transfection had worked (Table 5.1).

Selection medium containing Hygromycin (200µg/ml) was added to the cells 48h post transfection and also to control cells that had not been transfected. After 14 days selection all cells were dead on the control plates (cells not transfected with the PGKHyg) and colonies from the other plates were picked (Section 2.2.4). 21 colonies

of cells that had been transfected with pBI-EGFP*LMNAIR and PGKHyg were tested for tet inducible expression. Each cell line was seeded into 2 wells of a 6-well plate (2×10^5 cells per well) and doxycycline ($1\mu\text{g/ml}$) was added to the medium in one of the two wells. In the presence of doxycycline *GFP* expression should be 'switched on'. Therefore cells were looked at for expression of GFP using fluorescence microscopy 48h after doxycycline addition. Four of the cell lines tested expressed GFP when doxycycline was added to their medium (293 Tet-On LMNAIR) (Figure 5.13). One of these cell lines (1.2) also had low levels of GFP expression when no doxycycline was present. Ten cell lines that had been transfected with pBI-EGFP and PGKHyg were also tested for GFP expression. Four of these cell lines were shown to have GFP expression in the presence of doxycycline and were used as control cell lines for subsequent analysis. Four cell lines that had been transfected with just the PGKHyg were also used as controls. One reason why a large number of the colonies picked did not express GFP could be due to the integration of the pBI-EGFP vector into inactivated DNA. Alternatively these cells lines may contain PGKHyg but not the pBI-EGFP vector.

Transfection	PGKHyg	pBI-EGFP*LMNAIR	pBI-EGFP	pTP6
1	2 μg	2 μg	----	----
2	2 μg	----	2 μg	----
3	4 μg	----	----	----
4	----	----	----	4 μg

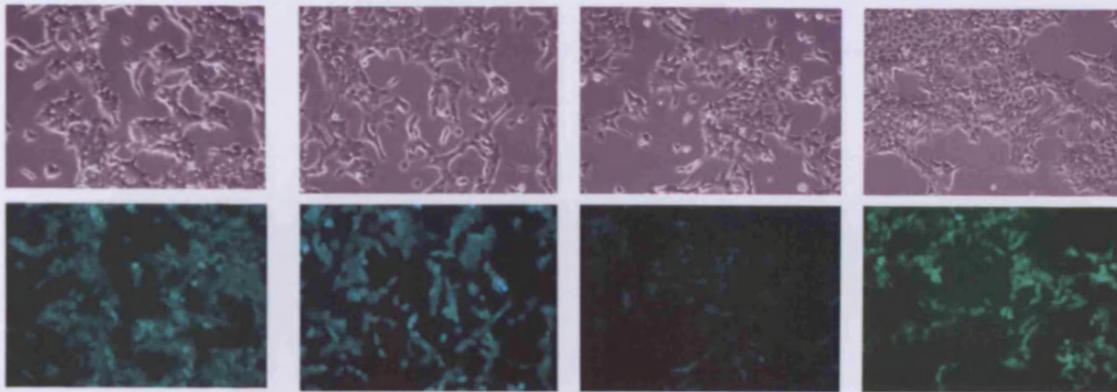
Table 5.1. Plasmids transfected into HEK 293 Tet-On cells using the calcium phosphate method (Section 2.2.2b).

RNA was extracted from the four cell lines of 293 Tet-On LMNAIR and the four lines of each of the control cells (293 Tet-On pBI-EGFP and 293 Tet-On PGKHyg) cultured with and without doxycycline (Section 2.3.2). RNA was DNase treated to remove any contaminating genomic DNA (Section 2.3.3) then reverse-transcribed to cDNA (Section 2.3.4). Semi-quantitative PCR analysis was performed on the cDNA using primers for human beta actin (*ACTB*) (control) (HActb_F and R), *GFP* (reporter gene) (EGFP_F and R) and *LMNA* (LMNA_F and R) (Table 2.7) (Section 2.3.5). There was seen to be expression of *GFP* in all cell lines that had been transfected with

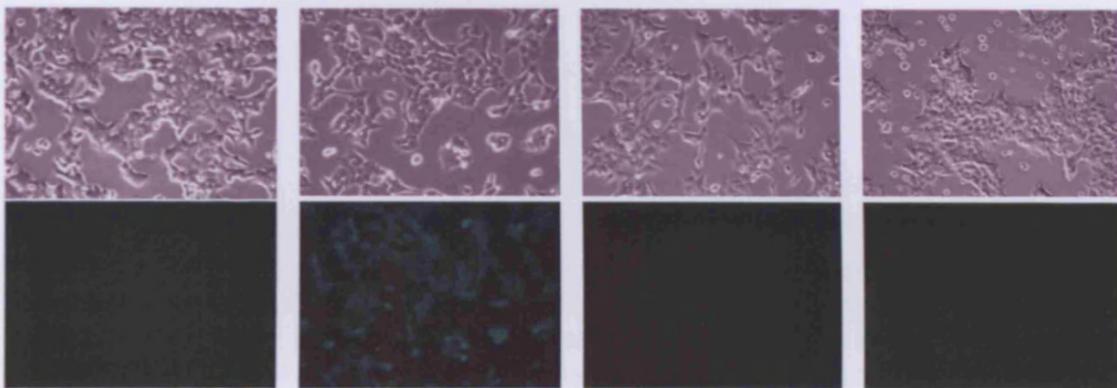
either the pBI-EGFP*LMNAIR or pBI-EGFP when doxycycline was present in the medium as expected and had been observed in the cells (Figure 5.13). There was also GFP expression in cell line 1.2 cultured in the absence of doxycycline that had been seen when cells were photographed. This could have been due to the site of integration of the vector in these cells. Insertion of the pBI-EGFP vector near an enhancer could lead to basal levels of EGFP expression even in the absence of doxycycline as was observed. It was predicted that cell lines that contained the pBI-EGFP*LMNAIR cultured with doxycycline would have a reduction in *LMNA* compared to culturing the same cells without doxycycline. There may have been a slight reduction of in *LMNA* in cell line 1.3 (Figure 5.13B). This could be confirmed using Real-Time PCR so that differences in the level of mRNA could be calculated. There was no observed reduction in *LMNA* levels by semi-quantitative PCR in the other cell lines when the cells were cultured with doxycycline.

The absence of *LMNA* knockdown in some cell lines could have been due to the inverted repeat not being expressed. Therefore RT-PCR analysis was performed on RNA extracted from the 293 Tet-On cell lines cultured with doxycycline to look for presence of the hairpin transcript. RNA from two of the 293 Tet-On LMNAIR (1.1, 1.4) and also two control cell lines (2.1 and 3.1) that had been DNase treated was reverse transcribed (Section 2.3.4). Instead of using oligo dT as the primer two oligonucleotides that anneal within the inverted repeat transcript were used Linker R and LMNA hp_R (Table 2.7). This was to specifically reverse transcribe the *LMNA* hairpin transcript. PCRs were then set up to amplify half of the inverted repeat transcript as attempts to PCR at either end of the inverted repeat had previously failed (Figure 5.9). One of the PCRs set up using primers LMNA hp_R and Linker F (Table 2.7) gave PCR products of the correct size (Figure 5.14). These were present in the 293 Tet-On LMNAIR cell lines (1.1 and 1.4) but not present in the control cell lines that contain the pBI-EGFP vector (2.1) or the PGKHyg vector only (3.1). Also the product was found to be present only in the 293 Tet-On LMNAIR 1.4 cells cultured with doxycycline thereby suggesting the *LMNA* IR repeat transcript is expressed only in the presence of doxycycline in this cell line. The PCR product from 1.4 (+ doxycycline) was sequenced confirming that it is specific for the *LMNA* IR sequence (Figure 5.15).

A) 293 Tet-On LMNA IR Cell lines cultured with doxycycline



293 Tet-On LMNA IR Cell Lines cultured in normal medium (no doxycycline)



1.1

1.2

1.3

1.4

B)

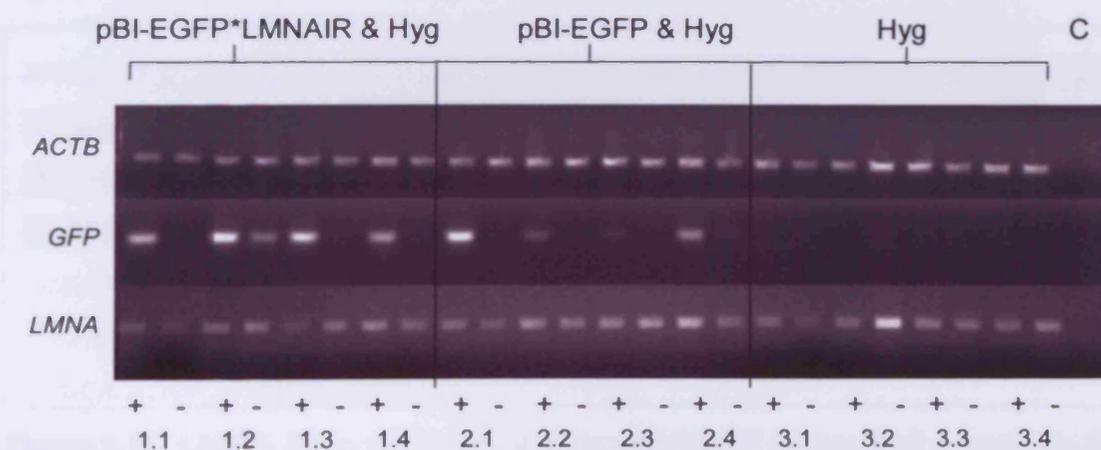


Figure 5.13. 293 Tet-On cells stably transfected with pBI-EGFP*LMNAIR cultured with (+) and without (-) doxycycline. A) Photographs of the cell lines. B) PCRs of cDNA from the cell lines including controls, 2.1-2.4 transfected with unmodified pBI-EGFP and selection vector, 3.1-3.4 transfected with hygromycin selection vector only. GFP was observed in cell lines 1.1 to 2.4 cultured with doxycycline as expected. GFP expression was also observed in 1.2 cultured without doxycycline therefore this cell line was not used for subsequent analysis.

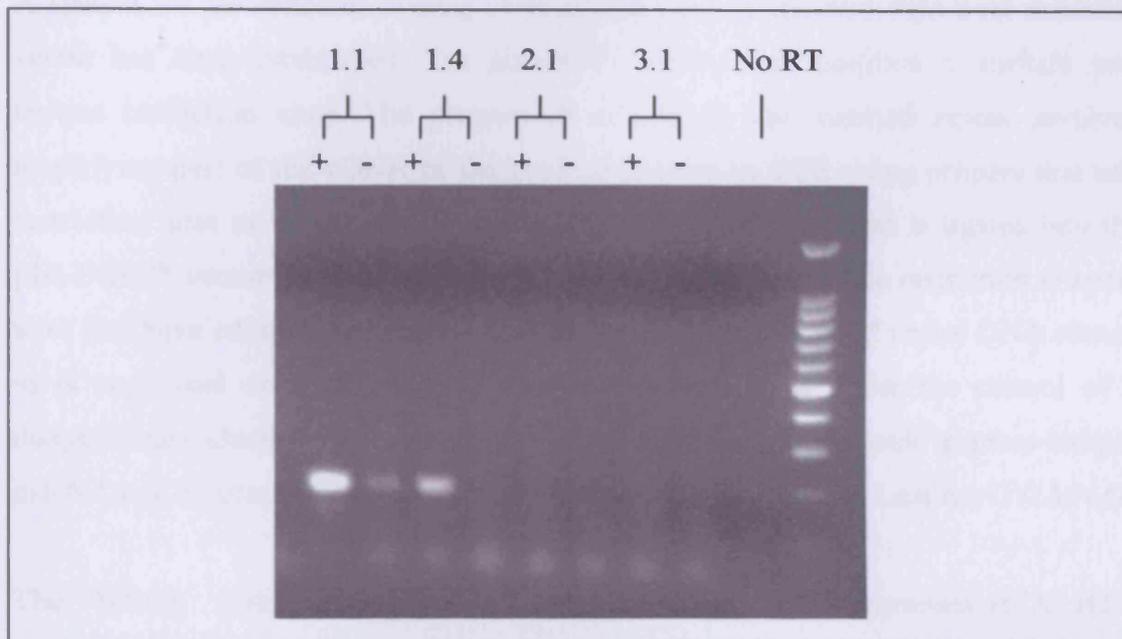


Figure 5.14. Confirmation that the inverted repeat was expressed in the 293 Tet-On LMNAIR cell lines cultured with doxycycline as studied by RT-PCR analysis. The primers used in this PCR reaction prime within the LMNA IR transcript. A PCR product of the expected size was observed in cell lines 1.1 and 1.4 cultured with doxycycline (+) with no expression seen in the control cell lines (2.1 and 3.1) that do not contain the pBI-EGFP*LMNA IR vector. There was also a PCR product in cell line 1.1 cultured in the absence of doxycycline that suggested there is background expression of the hpRNA transcript.

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ACTAGTATGAGATGCTGCGGCGGGTGGATGCTGAGAACAGGCTGCAGACCATGAAG
GAGGAACTGGACTTCCAGAAGAACATCTACAGTGAGGAGCTGCGTGAGACCAAGCG
CCGTCATGAGACCCGACTGGTGGAGATTGACAATGGGAAGCAGCGTGAGTTCGATG
GACCATTGCACTCGAGCGGACTTCGAACTCACGCTGCTTCCCATTGTCAATCTCCA
CCAGTCGGGTCTCATGACGGCGCTTGGTCTCACGCAGCTCCTCACTGTAGATGTTT
TTCTGGAAGTCCAGTTCCTCCTTCATGGTCTGCAGCCTGTTCTCAGCATCCACCCG
CCGCAGCATCTCATACTAGC
    
```

Figure 5.15. LMNA IR as transcribed from pBI-EGFP vector. Red sequence is the sequence of the LMNA inverted repeat with black sequence that of plasmid DNA. The highlighted region is the sequence of the PCR product from cell line 1.4 + doxycycline from Figure 5.14 and confirms that the LMNA hairpin is transcribed in these cells.

5.3. Discussion

A system for the efficient cloning of an inverted repeat of DNA into a tet inducible vector has been established. The pBI-EGFP vector was modified to include new unique restriction sites. The process of cloning in the inverted repeat involved amplifying part of the cDNA of the gene of interest by PCR using primers that add restriction sites on to the end of the fragment. Then the fragment is ligated into the pBI-EGFP* vector in two digestion and ligation steps that utilise restriction enzyme sites that have compatible cohesive end (Figure 5.7). The inverted repeat DNA cloned in is expressed as a hairpin dsRNA whose expression is under the control of a doxycycline inducible promoter. Two vectors were made that should express hairpin dsRNA one targeting mouse *Oct-4* and the other targeting human LaminA/C (*LMNA*).

The 'Tet-On' system requires a cell line that constitutively expresses rtTA. rtTA induces gene expression in the presence of doxycycline from a promoter that consists of a minimal promoter fused downstream of the tetO sequences (Figure 5.1). ES cell lines have been established that utilise the 'Tet-Off' system for transgene expression of *Oct-4* (Niwa *et al.*, 2000) and *Bcr-abl* (Era and Witte, 2000). The Tet-On system was chosen as doxycycline is only added to the cells when expression of the cloned gene is required. In this case addition of doxycycline would induce gene specific silencing at a defined time point.

It was not possible to make a mES cell line stably expressing rtTA and therefore the plasmid containing the inverted repeat for targeting *Oct-4* could not be tested for its ability to knockdown *Oct-4*. mES cells were electroporated with linearised pTet-On vector then cells containing the vector were selected for by the addition of neomycin. After 14 days selection cell lines were picked and expanded for analysis by transfection of pBI-EGFP that expresses GFP when rtTA and doxycycline are present. No GFP was observed in 21 mES cell lines tested. Cell lines were studied for rtTA expression by extraction of RNA and RT-PCR analysis. None of the cell lines tested were shown to be expressing rtTA (Figure 5.12). PCRs of genomic DNA that was extracted from the same cells confirmed that the pTet-On vector was not integrated in these cells (Figure 5.12).

During the attempts to make an rtTA expressing mES cell line a study was published that described failed attempts to make an ES cell line stably expressing a modified rtTA (rtTA2^S-S2) (Bryja *et al.*, 2003). rtTA2^S-S2 has a higher sensitivity to doxycycline therefore can be induced with lower levels of doxycycline than the original rtTA (Urlinger *et al.*, 2000). In the first of their experiments Bryja and colleagues used a vector expressing rtTA2^S-S2 (pNeoS2) from a CMV promoter that also had neomycin resistance gene that was expressed from a SV40 promoter. ES cells were electroporated with pNeoS2 and 13 clones of G418-resistant cells were picked after 10 days selection. When they were tested by transfection of a vector containing a tetracycline inducible luciferase none of the cell lines showed luciferase activity after the addition of doxycycline. Further analysis of gDNA from the cell lines confirmed none contain the rtTA2^S-S2. In a second experiment a new plasmid was made in which the expression of the neomycin resistance gene and the rtTA2^S-S2 were activated by the same promoter. No cells survived G418 selection unlike control cells only transfected with the vector containing the neomycin resistance gene. It was concluded that the rtTA2^S-S2 is toxic to mouse embryonic stem cells. Therefore in my study it was predicted that the cells that survived selection express the neomycin resistance gene but not the rtTA. This system is not suitable for inducible expression of hairpin dsRNA expression in mES cells at present.

Beyond the scope of this thesis it may be possible to test my vector using the Tet-Off system. This has been previously used in mES cells for the control of the expression of an *Oct-4* transgene (Niwa *et al.*, 2000) and for the inducible expression of Bcr-Abl, an activated tyrosine kinase oncogene (Era and Witte, 2000). In this case doxycycline would be added to the cells whilst establishing the mES Tet-Off cell line containing the pBI-EGFP*Oct-4IR. The withdrawal of doxycycline should induce hairpin dsRNA expression and knockdown of *Oct-4*. This approach should be suitable for studying gene expression in mES cells but is less suitable for establishment of Tet-Off inducible mice as the continued presence of doxycycline has been shown to be embryotoxic (Moutier *et al.*, 2003).

One inducible system, for gene specific silencing in mES cells by RNAi, uses the Cre-LoxP system (Coumoul *et al.*, 2004). A vector was made containing a loxP flanked

neomycin cassette inserted into the U6 RNA polymerase III promoter (pBS/U6-Neo). The presence of the neomycin cassette impairs U6 promoter activity. A pBS/U6-Neo vector was made that contains a shRNA targeting *Fgfr2*. This vector was transfected into a mouse ES cell line (D4) that was made that stably expresses the Cre recombinase Cre-ER^{T2}. Cre-ER^{T2} is a fusion between Cre and the G521R mutant of the human oestrogen receptor ligand binding domain (Feil *et al.*, 1997). Treatment of D4 ES cells with 4OH-tamoxifen (4HT) induces the Cre recombinase that exercises the neo cassette from the U6 promoter. This results in the expression of the *Fgfr2* hpRNA. A knockdown of *Fgfr2* was observed by RT-PCR analysis 24h after treatment of cells with 4HT with a large reduction (>90%) seen at 96h. This corresponded with a 30% decrease in growth of mES cells 120h after induction. Also the number and size of colonies was decreased. Similar results were obtained for a second gene they targeted, Survivin, that inhibits apoptosis and is also involved in the regulation of cell division. Therefore it was concluded that system could be used for studying function of genes essential for cell growth and viability.

In order to test the tet-inducible system expressing hairpin dsRNA using pBI-EGFP* the 293 Tet-On (human embryonic kidney) cell line was purchased (Clontech, Oxford, UK) that stably expresses rtTA. According to published studies shorter double-stranded RNA does not induce an interferon response in these cells. In one study using 293 cells a non-specific response was only observed when 500bp dsRNA targeting a reporter gene (luciferase) was transfected and not when 50bp dsRNA was transfected (Elbashir *et al.*, 2001a). Also in another study a plasmid expressing β -galactosidase and dsRNA targeting it were cotransfected. There was no observed reduction of β -galactosidase with the transfection of the dsRNA (Caplen *et al.*, 2000). Therefore 293 Tet-On cells were chosen from the Tet-On cell lines that were available as it was predicted dsRNA would not induce an interferon response in these cells.

Lamin A/C (*LMNA*) was chosen as the target gene for knockdown as previous studies have successfully knocked down this gene (Elbashir *et al.*, 2001a). The region of *LMNA* that was targeted (155bp) included a region that had been previously targeted for RNAi induced silencing using shRNAs (Elbashir *et al.*, 2001a). The vector constructed containing the inverted repeat of *LMNA* (pBI-EGFP**LMNAIR*) was

stably transfected into the 293 Tet-On cell line. GFP expression in cell lines cultured with doxycycline (Figure 5.12) suggests that the hairpin dsRNA is expressed as the GFP and hpRNA are expressed from a bidirectional tet inducible promoter (Figure 5.1). The hpRNA *LMNA* transcript was confirmed in the cell line 293 Tet-On 1.1 and 1.4 (cultured with doxycycline), yet a reduction in *LMNA* expression was not observed in these cell lines. This could have been because the *LMNA* hairpin was either not expressed properly or processed incorrectly. Alternatively the knockdown of *LMNA* may not have been detected by semi-quantitative PCR analysis. Further analysis using Real Time PCR would allow quantification of mRNA levels so that differences in the level of mRNA could be calculated.

Chapter 6

General Discussion

6. General Discussion

The aim of this project was to develop methods for inducing gene specific conditional silencing in mES cells using RNAi. At the beginning of the project it was known that dsRNAs could be used as a fast and efficient method for inhibiting gene function in *C. elegans* (Fraser *et al.*, 2000) and *Drosophila* (Misquitta and Paterson, 1999). Injection of dsRNA into mouse oocytes resulted in specific suppression of target genes (Wianny and Zernicka-Goetz, 2000). Also siRNAs had been shown as intermediates of the processing of dsRNA in the RNAi process and the transfection of these into mammalian cells (including human embryonic kidney 293 and HeLa cells) induced gene silencing (Elbashir *et al.*, 2001a).

6.1. Use of siRNAs for elucidating gene function in mES cells

In the initial experiments of this study the transfection of siRNAs into mES cells was shown to induce gene silencing of plasmid expressed MmGFP that was cotransfected with the MmGFP siRNA. The reduction of MmGFP expression was shown to be specific as cotransfection of the plasmid with a control siRNA duplex did not reduce GFP expression in the mES cells as observed by fluorescence microscopy and RT-PCR. Furthermore the MmGFP siRNA was also shown to specifically induce knockdown of endogenously expressed MmGFP.

Subsequently three siRNAs targeting Oct-4 were designed using the then published methods (Harborth *et al.*, 2001). Starting 75 bases down from the start codon of the cDNA gene sequence to be targeted an AA dimer was found and the next 19nt were recorded. The GC content of the sequence was calculated and if it was not between 50 and 70% another AA dimer was located and GC content calculated again. The sequence was subject to a BLAST-search against EST libraries to ensure only one gene was targeted. The three siRNAs were transfected into mES cells and two were found to reduce *Oct-4* expression and also induce differentiation of the mES cells down the trophectoderm lineage as shown by the expression of trophectoderm marker genes *Hand1* and *Cdx2*. Since the design of these siRNAs new design criteria have been established as more is known about the mechanism of RNAi and the

characteristics of functional siRNAs (Ding *et al.*, 2004; Reynolds *et al.*, 2004). These include having a GC content of 36-52%, lower internal stability in 9-14bp region (as starting from the 5' end of the antisense strand) and also the 5' end of the antisense strand should have a lower internal stability than the 5' end of the sense strand. The Oct-4 siRNAs that had been designed using the original methods were assessed with the new criteria using software based on these criteria (<http://sfold.wadsworth.org>). It was found that one of the Oct-4 siRNAs was predicted to be non-functional and that it was the duplex that had been shown to be non-functional in the transfection experiments. Therefore in future experiments this software would be used to increase the likelihood of designing functional siRNAs.

Transfection of siRNAs was shown to induce gene silencing but as not all cells were transfected only a few showed a phenotype. Another limitation of these experiments is that those cells taking up the siRNA resulting in a change in phenotype may be 'selected against' as untransfected cells have a growth advantage. Therefore methods for identification or selection of the cells transfected with the siRNAs are needed. The use of labelled siRNAs would allow a change in cell phenotype to be directly attributed to the presence of the siRNA in the cell. Also it may be possible to enrich for the labelled siRNA transfected cells by cell sorting using a fluorescence-activated cell sorter (FACS).

RNAi induction was shown to be possible in mES cells but reductions in gene expression are transient when using siRNAs. In order to look at long term silencing effects, the use of plasmids that stably express shRNAs was investigated.

6.2. Transfection of plasmids expressing shRNA into mES cells

Three genes were targeted using vectors expressing hpRNA. These were *Rex-1*, Laminin B1 (*Lamb1*) and *Oct-4*. The vector used for cloning in invert repeat oligonucleotides for *Rex-1* and *Lamb1* contain a *Sh ble* gene conferring ZeocinTM resistance. Therefore all of the cells that survived selection should express the shRNA and therefore display knockdown of the target gene. *Lamb1* expression was reduced in the stably psiRNA_*Lamb1* transfected cell lines as analysed by RT-PCR. In psiRNA_*Rex-1* cell lines that had survived selection only one cell line had a reduction

in *Rex-1*. It was predicted that this is because a reduction in *Rex-1* would have resulted in a change in phenotype of the cell that would have been selected against. The cell line that was selected may be expressing enough *Rex-1* so that cells do not differentiate and can be maintained in culture.

In order to study the immediate knockdown effect of the *Rex-1* shRNA on ES cells the transfection experiment designed using the psiOct-4 vector could be performed. The psiRNA-*Rex-1* vector would be cotransfected with a GFP expressing vector into mES cells. Photographs of transfected cells (GFP expressing) would be taken for analysis of cell size 48h post transfection. In the control experiment the psiRNA-*Lamb1* vector and GFP expressing vector would be cotransfected. It is predicted that a reduction in *Rex-1* by shRNA expression would result in the differentiation of cells and therefore an increase in cell size. The system for the psiOct-4 vector could be used to study other genes that may be involved in maintaining the pluripotency of mES cells. A reduction of gene expression of these genes is predicted to result in the differentiation of cells.

The CCB psiRNA_*Rex-1* cell line and CCB psiRNA_*Lamb1* knockdown cell lines that were made could be investigated further. Studies of gene expression of other genes typically expressed in mES cells or markers of differentiation could be studied. The CCB psiRNA_*Rex-1* cell line and CCB psiRNA_*Lamb1* could be differentiated and differences in phenotype and gene expression as compared to control cells studied.

The levels of *Lamb1* and *Rex-1* were reduced but there was still some RNA expressed that may be enough to make adequate protein. This is an intrinsic disadvantage of the RNAi system despite the success in making stable expression cell lines. There was no phenotype observed in the CCB psiRNA_*Rex-1* cell line as was predicted this could be due to the levels of *Rex-1* present being sufficient to maintain pluripotency of mES cells. Alternatively knockdown of this gene may not induce a phenotypic effect and therefore there is no easy way of assessing the knockdown. Cell lines that had a greater knockdown of *Rex-1* and a resulting change in phenotype are likely to be selected against. The ability to induce silencing once a cell line was established that contains the hairpin RNA would be an advantage as it overcomes the problem of

'selecting against' stably transfected cell lines. Therefore a tetracycline inducible system was investigated for inducible gene silencing.

6.3. Inducible systems for gene specific silencing by RNAi

A system was developed for efficient cloning of an inverted repeat into a tetracycline-inducible vector. The advantage of using such a system is cells that contain the hairpin expression vector can be established before knocking down the target gene. In the case of this study the Tet-On system was investigated whereby the expression of the hairpin dsRNA is switched on when doxycycline is added to the medium. The tet-inducible plasmid also expresses GFP from the same tet inducible promoter allowing identification of cells that contain the vector.

A vector was designed for the efficient cloning of an inverted repeat of the target gene. The original vector constructed, designed to target *Oct-4* could not be tested as attempts to make a mES 'Tet-On' cell line expressing rtTA failed. A study published since has shown that the rtTA is toxic to mES cells and therefore it is not possible to establish cell lines stably expressing rtTA (Bryja *et al.*, 2003). A second vector was made containing an inverted repeat of part of the LaminA/C (*LMNA*) gene (pBI-EGFP_LMNAIR) whose expression was under the control of the tetracycline responsive promoter. This vector was tested in the commercially available human embryonic kidney cell line (HEK 293) Tet-On. This cell line stably expresses rtTA. The pBI-EGFP_LMNAIR vector was transfected into these cells. The cell lines were shown to contain the vector by the expression of GFP when doxycycline was added to the cells. There was no observable decrease in *LMNA* expression by semi-quantitative PCR analysis although the *LMNAIR* transcript was shown to be expressed.

The hairpin dsRNA was designed to target a 155 nt region of the *LMNA* coding region and also included a 21 nt sequence that had previously been targeted by a functional shRNA (Elbashir *et al.*, 2001a; Koper-Emde *et al.*, 2004). Recombinant human Dicer has been shown to cleave dsRNA into ~22 nt fragments progressively from their termini (Zhang *et al.*, 2002). It was also shown that dsRNA as small as 30bp are efficiently cleaved by Dicer. Therefore it is predicted that at least six siRNAs would be produced from the *LMNA* hairpin dsRNA. It is possible that the Dicer produced

fragments may not include the validated siRNA sequence or any functional siRNAs. The use of a longer hairpin RNA would generate more siRNAs against the target gene but may also induce non-specific effects due to an interferon response. In one study a non-specific response was observed by transfection of 500bp dsRNA but not 50bp dsRNA into 293 cells (Elbashir et al., 2001a). Although transfection of a 472bp dsRNA into HEK 293 cells was shown to induced gene specific silencing in another study (Morimoto *et al.*, 2004). Alternatively the knockdown of *LMNA* may not have been detected by semi-quantitative PCR analysis. Further analysis using Real Time PCR would allow quantification of mRNA levels so that differences in the level of mRNA could be calculated.

The Tet-Off system has been previously used in mES cells for the control of the expression of an *Oct-4* transgene (Niwa *et al.*, 2000). A Tet-Off system was also established for the inducible expression of *Bcr-Abl*, an activated tyrosine kinase oncogene (Era and Witte, 2000). Therefore in the future establishment of a Tet-Off cell line may enable the inducible hairpin dsRNA vector targeting *Oct-4* to be tested. In this case doxycycline would be added to the cells whilst establishing a mES Tet-Off cell line containing the *Oct-4* inverted repeated. Withdrawal of doxycycline induces hairpin dsRNA expression. This approach should be suitable for studying gene expression in mES cells but is less suitable for establishment of Tet-Off inducible mice as the continued presence of doxycycline has been shown to be embryotoxic (Moutier *et al.*, 2003).

Alternatively, use of other inducible systems could also be considered in this context. The Cre-LoxP system has been used for inducible gene specific silencing in mES cells (Coumoul *et al.*, 2004). A vector was made containing a loxP flanked neomycin cassette inserted into the U6 RNA polymerase III promoter (pBS/U6-Neo). The presence of the neomycin cassette impairs U6 promoter activity. shRNA is expressed from the U6 promoter when Cre recombinase is induced and the neomycin cassette is excised from the promoter. Several shRNAs may need to be tested before a suitable one for inducing gene silencing is found.

6.4. Current literature on the use of RNAi for studying gene function in mES cells and mouse models

From these experiments it can not be concluded that RNAi is 'fast, efficient' method for studying ES cell gene function. Transfection of siRNAs into cells resulted in a inhomogeneous population of cells, only some with observable knockdown. Vectors stably expressing shRNA were tested to overcome this problem. Selected cell lines should contain the vector and therefore display knockdown. The problem of using this method for studying genes involved in pluripotency is that cells containing knockdown of a gene are likely to have a growth disadvantage and be selected against. The cotransfection of a vector expressing GFP with the shRNA expressing vector allows the effect of the knockdown to be analysed on a cell by cell basis as demonstrated in the use of the psiOct-4 vector. In this case 100 cells were measured from control and shRNA expressing cells but this is a time consuming process and would not be possible to perform on a large scale unless suitably automated. An efficient method for cloning an inverted repeat into the pBI-EGFP* vector has been demonstrated. The coexpression of EGFP with the hairpin dsRNA would also act as a marker for transfected cells. This use of this vector could not be demonstrated as rtTA has been shown to be toxic to mES cells (Bryja *et al.*, 2003).

At present there are only 11 published papers describing the successful use of RNAi for gene silencing in mES cells (Table 6.1). Only 5 of these studies describe the knockdown of targets other than Oct-4 and GFP. Knockdown of *Oct-4* causes differentiation of cells and presence (or absence) of GFP can be visualised by fluorescence microscopy. Some of the other genes were also chosen as the function of the gene was known in mES cells and therefore were used in order to test the particular system of RNAi. *Rasa1* knockdown confirmed what had already been demonstrated in derived *Rasa1* homozygous null mES cells (Kunath *et al.*, 2003). Knockdown of *Dnmt* in mES cells was compared to characteristics of mES cells mutant for *Dnmt* had already been reported (Li *et al.*, 1992; Ventura *et al.*, 2004). It was predicted that knockdown of *Fgfr2* would reduce cell proliferation as had been shown in other cell types (Coumoul *et al.*, 2004). Also they already had *Fgfr2* knockout mice to compare any phenotype produced by the presence of shRNA *in vivo*. Therefore the studies to date have demonstrated that RNAi strategies are

possible for gene specific silencing but the methods have yet to show their worth for determining the function of novel genes.

Traditional methods for controlling gene expression include the expression of transgenes, gene knockout by homologous recombination, and the production of dominant negative proteins (Houdebine, 2002). These methods can introduce dominant gain of function, complete loss of function, or dominant loss of function of the target protein. In RNAi gene expression is reduced but residual levels of target gene expression and target protein remain (Carmell and Hannon, 2004). Using other methods low levels of RNA may be observed but this RNA usually includes a resistance cassette or a mutation within the sequence and therefore any protein produced is non-functional. Conversely residual RNA expression that escapes RNAi is in no way disrupted and can produce protein, levels of which may be enough to rescue the phenotype.

Genes Targeted	Method of inducing RNAi	Notes	Reference
<i>GFP</i>	Transfection of dsRNAs or a vector expressing hairpin dsRNA (547bp).	GFP expression was decreased by up to 70% as measured by fluorescence of cell lysate but had 'recovered' by day 5 after transfection.	(Yang <i>et al.</i> , 2001)
<i>GFP</i>	Transfection of shRNA expressed from an RNA polymerase II promoter.	Construct was transcribed does not have a polyA but it is likely a 7MeG 5' cap is added. Transcript should have five unpaired residues at its 3' end and a 13 nt 5' overhang.	(Grabarek <i>et al.</i> , 2003)
<i>Rasal</i> (<i>RasGAP</i>)	Electroporation of shRNA expressing vector from a H1 RNA polymerase III promoter.	12 drug resistant ES cell lines were established. Protein levels, analysed by Western blotting, showed a significant reduction in RasGAP in 11 cell lines and only a slight reduction in the other cell line.	(Kunath <i>et al.</i> , 2003)
<i>CD8</i> , <i>p53</i> , a neuron specific isoform of Mena	Lentiviral based vector that expresses shRNA from a U6 RNA polymerase III promoter.	The vector also expresses EGFP as a reporter gene. GFP expressing mES cells were selected by cell sorting and injected into blastocysts.	(Rubinson <i>et al.</i> , 2003)
<i>EGFP</i> , <i>Oct-4</i> .	Transfected vector expressing shRNA from U6 or H1 RNA polymerase III promoter	The vector also contained an independently expressed dsRed reporter gene. Cells were FACS sorted by dsRed expression on day 1.5 to enrich the population.	(Velkey and O'Shea, 2003)
<i>Oct-4</i>	Transfection of Dicer-produced pools of siRNAs.	Transfected cells differentiated to trophectoderm like cells.	(Carpenter and Zernicka-Goetz, 2004)
<i>Fgfr2</i> , <i>Survivin</i> .	Transfection of vector that expresses shRNA under an inducible system that uses the Cre-LoxP system	The mouse U6 RNA polymerase III promoter has a loxP flanked neomycin cassette that prevents expression of the shRNA. A tamoxifen inducible cre construct was used to remove the neomycin cassette allowing expression the shRNA.	(Coumoul <i>et al.</i> , 2004)

Table 6.1. Studies of gene expression in mouse embryonic stem cells using RNAi.

Genes Targeted	Method of inducing RNAi	Notes	Reference
<i>Oct-4</i>	Transfection of restriction enzyme generated siRNAs (REGS)	Six sense and antisense targeting <i>Oct-4</i> by the REGS method. Three of these sense strand sequences were shown to induce gene specific silencing of <i>Oct-4</i> .	(Sen <i>et al.</i> , 2004)
<i>GFP</i>	Transfection of plasmid expressing shRNA from a mouse U6 promoter	GFP expression study by flow cytometry.	(Tang <i>et al.</i> , 2004)
<i>Dmrt1</i>	Transfection of lentiviral based vector that expresses shRNA from U6 promoter.	Two vectors were constructed for conditional cre-lox regulated RNAi.	(Ventura <i>et al.</i> , 2004)
<i>EGFP, Oct-4</i>	Transfection of siRNAs	FACS analysis showed GFP expression was reduced in 97% of transfected cells.	(Takada <i>et al.</i> , 2005)

Table 6.1. Studies of gene expression in mouse embryonic stem cells using RNAi (continued).

Non specific effects have been observed in the use of shRNAs where gene expression of genes other than the target is reduced. These off target effects may occur when shRNAs target an mRNA for degradation that is partly homologous to the shRNA. It has been shown that some mismatches between the target sequence and the shRNAs can be tolerated and still result in a reduction in gene expression. Off target effects have been shown to be induced by siRNAs with as few as 11 nt of complementarity to mRNA (Jackson *et al.*, 2003). It has also been shown that mismatches between the target mRNA and up to 4 nt residues at the 5' end or the 3' terminus of the siRNA incorporated into RISC do not result in complete loss of cleavage activity (Martinez and Tuschl, 2004). Therefore in order to demonstrate the phenotype observed in mES cells expressing shRNA is due to the reduction of the target gene, at least two independent cell lines would need to be made each targeting a different region of the gene.

Other off target effects include the induction of an interferon response (Bridge *et al.*, 2003). Human lung fibroblasts were infected with the lentiviral vector containing a shRNA expression cassette that reduced target gene expression to 3% of the normal level. Microarray analysis identified induction of interferon target genes as well as a reduction in gene expression of the targeted gene. Transfection of siRNA corresponding to the lentiviral produced shRNA also triggered an interferon response showing that it was not due to the presence of the lentivirus. In another study Sledz and colleagues studied the non-specific effects of siRNA transfection into cells (Sledz *et al.*, 2003). siRNAs targeting Lamin A/C and GAPDH were transfected into a human glioblastoma line. A siRNA dose-dependent decrease in the target gene was observed as expected but there was also an increase in the expression of interferon-stimulated genes. In the use of mES cells expressing transgenes or dominant negative proteins the location of the genetic modification is known and stable. Therefore any change in phenotype can be attributed to the presence of the mutation.

The difficulties of establishing beyond doubt the phenotypic consequences of RNAi have also been demonstrated *in vivo*. The generation of transgenic RNAi mice using vectors expressing shRNAs has been shown by several groups (Table 6.2). One of the limitations of using shRNAs for the production of transgenic mice is that shRNAs can

cause different levels of knockdown of the target gene or no affect at all depending on the region of the mRNA targeted. Carmell and colleagues were unsuccessful in producing transgenic mice using shRNA expressing vectors targeting 7 different genes (Carmell *et al.*, 2003). Three different shRNA constructs were designed for each gene and each was injected into the pronuclei to produce transgenic founder mice. The targeted genes were chosen as the expected phenotype was known. None of the animals produced showed distinct or reproducible phenotypes. In a different approach by the same group the ability of the expressed shRNA to induce gene specific gene silencing was assessed in mES cells (Carmell *et al.*, 2003). A shRNA expressing vector targeting the 5' end of *Neil1* coding region was electroporated into mES cells. The knockdown of the target gene and protein were confirmed. Two independent mES cell lines that had a ~80% reduction in *Neil1* protein due to the presence of the shRNA construct were injected into blastocysts. Chimeras were outcrossed and germline expression was observed. *Neil1* levels measured in liver extracts were shown to be reduced by the same amount as in the ES cell lines from which the mice were made. Therefore in this case assessment of the level of knockdown induced by an individual shRNA could be assessed in mES cells to ensure that the expression of the target gene is reduced in transgenic mice produced using these cells; although this would limit targeting by RNAi to gene expressed in mES cells. Also in RNAi induced gene silencing the levels of mRNA expression are reduced but some protein is still expressed. This could be enough to maintain the wildtype phenotype *in vivo*.

The aim of this thesis was to establish methods for studying gene function in mES cells using RNAi. The methods demonstrated in this thesis have not shown RNAi to live up to its promise of 'quick and efficient' method for establishing gene function. At present most other mES cell studies (Table 6.1) have only confirmed what is already know about gene function. Establishing the function of novel genes may be more difficult and the production of knockout mice is still the best option particularly using facultative deletion e.g. cre or tamoxifen methods.

Gene targeted	Method	Notes	Ref
<i>EGFP</i>	Injection of a vector that expresses shRNA targeting GFP into the pronuclear stage of fertilised mouse eggs. (vector also expresses red fluorescent protein)	GFP was measured in embryos by immunoblotting. GFP expression was found to have been reduced to between 4 and 24% of the control level in the embryos containing the GFP shRNA expressing vector as compared to the control. Looking at individual organs, 'the silence affect was nearly complete' in the heart and pancreas. The brain, liver, kidney and skin also had reduced fluorescence.	(Hasuwa <i>et al.</i> , 2002)
<i>Neill</i>	Electroporation of a vector expressing shRNA into mES cells. Stable cell lines selected then injected into blastocysts.	ES cell lines showed a ~80% reduction of NEIL1 protein. A similar level knockdown was observed <i>in vivo</i> .	(Carmell <i>et al.</i> , 2003)
<i>Rasal</i> (<i>RasGAP</i>)	Electroporation of shRNA expressing vector from a H1 RNA polymerase promoter. Embryos were produced using the tetraploid aggregation method.	Embryos were dissected at embryonic day 9.5. Embryos derived from the ES cell line with only a slight decrease in RasGAP expression were the same as the wildtype. Embryos produced from three ES cell lines that had significantly reduced RasGAP had phenotypes similar to that of the null phenotype.	(Kunath <i>et al.</i> , 2003)
<i>CD8</i> , <i>CD25</i> , <i>p53</i> <i>Mena</i> ⁺	Use of lentiviral vector containing a shRNA expression cassette. mES cells stably expressing hpRNA targeting CD8 were injected into blastocyst. In the case of CD8, CD25, p53 and Mena ⁺ transgenic animals were generated by direct lentiviral infection of single cell embryos.	The lentiviral vector also contained GFP expressed from an independent promoter. Chimeric mice were shown to have GFP and shRNA expression in all tissues tested.	(Rubinson <i>et al.</i> , 2003)

Table 6.2. Mouse models made using RNAi technology.

Gene targeted	Method	Notes	Reference
<i>Ski</i> (transcriptional corepressor)	Injection of a vector that expresses long dsRNA (540bp) that lacks the 5'-cap structure and the 3'-poly(A) tail into fertilised mouse oocytes.	Histological analysis. Mice embryos that expressed the hairpin dsRNA targeting <i>Ski</i> had phenotypes that were similar to those of <i>Ski</i> -deficient embryos.	(Shinagawa and Ishii, 2003)
<i>EGFP</i>	Transduction of lentiviral vector expressing shRNA from H1 promoter into blastocysts.	A reduction in GFP was seen in blastocysts and the resulting progeny also showed knockdown of GFP.	(Tiscornia <i>et al.</i> , 2003)
<i>CD8, p53</i>	Used a cre-lox conditional vector that expresses shRNA to infect mES cells. Three ES cell lines were used for generating conditional knockdown mice.	Transmission of the vector was observed in progeny for two of the ES cell clones used.	(Ventura <i>et al.</i> , 2004)

Table 6.2. Mouse models made using RNAi technology (Continued)

6.5. Conclusion

Since the discovery of RNAi in *C. elegans* 7 years ago there have been many advances in the understanding of the mechanism by which dsRNA induces gene specific silencing (Fire *et al.*, 1998). Injection of dsRNAs into mouse oocytes and preimplantation embryos and transfection of dsRNA into mES cells were all demonstrated to induce specific gene silencing (Svoboda *et al.*, 2000; Wianny and Zernicka-Goetz, 2000; Yang *et al.*, 2001). The transfection of dsRNAs into mammalian somatic cell lines did not induce potent and specific gene silencing (Caplen *et al.*, 2000; Ui-Tei *et al.*, 2000). The interferon response present in these cell lines led to non-specific mRNA degradation and inhibition of protein synthesis. The discovery of siRNAs as intermediates in the processing of the dsRNA allowed the development of methods for RNAi induction in mammalian cell lines as these do not trigger an interferon response (Elbashir *et al.*, 2001a).

RNAi has shown to be an invaluable tool for determining gene function in *C. elegans* and *Drosophila*. RNAi libraries targeting all of the predicted genes in mouse, rat and human cells combined with the use of a large number of cell based assays allows high throughput screening of gene function in cell lines (Cullen and Arndt, 2005). These RNAi libraries have been used to identify genes various cell pathways including the p53 pathway (Berns *et al.*, 2004) in human fibroblast cells and those involved in proteasome function in protein degradation (Paddison *et al.*, 2004) in HEK 293T cells.

The use of RNAi has been more successful for studying gene function in lower organisms such as *Drosophila* and *C. elegans*. This is because dsRNA can be used for inducing gene silencing in these organisms whereas dsRNA induces an interferon response in mammalian cell lines. The use of siRNAs has overcome this to some extent, but not all are functional unlike dsRNA that targets across the mRNA. Also in the case of *C. elegans* there is amplification of the RNAi silencing effect through the action of an RNA-dependent RNA polymerase (RdRP) (Sijen *et al.*, 2001). The RdRP converts the target mRNA into dsRNA that can be cleaved by Dicer. This produces new pools of siRNA that silence the expression of the target gene. This amplification of RNAi has not been shown in mammalian cells. The induction of RNAi in *C.*

elegans using dsRNA is relatively easy. For example worms can be soaked in dsRNA solution (Tabara *et al.*, 1998) or fed dsRNA expressing bacteria (Maeda *et al.*, 2001). Delivery systems for siRNAs into mammalian cells present more of a challenge. The affect of RNAi induced knockdown of gene expression in *C. elegans* can easily be scored by observations of embryonic lethality, morphological defects and retarded growth using a dissecting microscope. Conversely, scoring mammalian cells phenotypically presents more of a challenge.

In this study the objective was to develop methods for studying gene function in mES cells utilising RNAi. The methods demonstrated in this thesis have shown RNAi induced silencing of 3 genes expressed in mES cells. Improvements to the design and delivery of effective siRNAs and the development of conditional systems for the expression of shRNAs will improve the efficiency of RNAi in mES cells. In the future the use of RNAi libraries has the potential to increase the throughput of RNAi for studying mES cell gene function with the use of suitable reporter systems.

Bibliography

Bibliography

- (2004) Finishing the euchromatic sequence of the human genome. *Nature*, **431**, 931-945.
- Ambrosetti, D.C., Scholer, H.R., Dailey, L. and Basilico, C. (2000) Modulation of the activity of multiple transcriptional activation domains by the DNA binding domains mediates the synergistic action of Sox2 and Oct-3 on the fibroblast growth factor-4 enhancer. *J Biol Chem*, **275**, 23387-23397.
- An, D.S., Xie, Y., Mao, S.H., Morizono, K., Kung, S.K. and Chen, I.S. (2003) Efficient lentiviral vectors for short hairpin RNA delivery into human cells. *Hum Gene Ther*, **14**, 1207-1212.
- Anneren, C., Cowan, C.A. and Melton, D.A. (2004) The Src family of tyrosine kinases is important for embryonic stem cell self-renewal. *J Biol Chem*, **279**, 31590-31598.
- Aubert, J., Dunstan, H., Chambers, I. and Smith, A. (2002) Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. *Nat Biotechnol*, **20**, 1240-1245.
- Avilion, A., A., Nicolis, S., K., Pevny, L., H., Perez, L., Vivian, N. and Lovell-Badge, R. (2003) Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes & Development*, **17**, 126-140.
- Barton, G.M. and Medzhitov, R. (2002) Retroviral delivery of small interfering RNA into primary cells. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 14943-14945.
- Beck, F., Erler, T., Russell, A. and James, R. (1995) Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. *Dev Dyn*, **204**, 219-227.
- Ben-Shushan, E., Thompson, J.R., Gudas, L.J. and Bergman, Y. (1998) Rex1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. *Molecular and Cellular Biology*, **18**, 1866-1878.
- Berns, K., Hijmans, E.M., Mullenders, J., Brummelkamp, T.R., Velds, A., Heimerikx, M., Kerkhoven, R.M., Madiredjo, M., Nijkamp, W., Weigelt, B., Agami, R., Ge, W., Cavet, G., Linsley, P.S., Beijersbergen, R.L. and Bernards, R. (2004) A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature*, **428**, 431-437.
- Bernstein, E., Caudy, A.A., Hammond, S.M. and Hannon, G.J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature (London)*, **409**, 363-366.

- Bernstein, E., Kim, S.Y., Carmell, M.A., Murchison, E.P., Alcorn, H., Li, M.Z., Mills, A.A., Elledge, S.J., Anderson, K.V. and Hannon, G.J. (2003) Dicer is essential for mouse development. *Nat Genet.*
- Billy, E., Brondani, V., Zhang, H., Muller, U. and Filipowicz, W. (2001) Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 14428-14433.
- Bradley, A., Evans, M., Kaufman, M.H. and Robertson, E. (1984) Formation of germline chimaeras from embryo-derived teratocarcinoma cell lines. *Nature*, **309**, 255-256.
- Bridge, A.J., Pebernard, S., Ducraux, A., Nicoulaz, A.L. and Iggo, R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet.*, **34**, 263-264.
- Brook, F.A. and Gardner, R.L. (1997) The origin and efficient derivation of embryonic stem cells in the mouse. *Proc Natl Acad Sci U S A*, **94**, 5709-5712.
- Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science (Washington D C)*, **296**, 550-553.
- Bryja, V., Pachernik, J., Kubala, L., Hampl, A. and Dvorak, P. (2003) The reverse tetracycline-controlled transactivator rtTA2s-S2 is toxic in mouse embryonic stem cells. *Reprod Nutr Dev*, **43**, 477-486.
- Caplen, N.J., Fleenor, J., Fire, A. and Morgan, R.A. (2000) dsRNA-mediated gene silencing in cultured Drosophila cells: A tissue culture model for the analysis of RNA interference. *Gene (Amsterdam)*, **252**, 95-105.
- Caplen, N.J., Parrish, S., Imani, F., Fire, A. and Morgan, R.A. (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 9742-9747.
- Capodici, J., Kariko, K. and Weissman, D. (2002) Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *Journal of Immunology*, **169**, 5196-5201.
- Carmell, M.A. and Hannon, G.J. (2004) RNase III enzymes and the initiation of gene silencing. *Nat Struct Mol Biol*, **11**, 214-218.
- Carmell, M.A., Xuan, Z., Zhang, M.Q. and Hannon, G.J. (2002) The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev*, **16**, 2733-2742.
- Carmell, M.A., Zhang, L., Conklin, D.S., Hannon, G.J. and Rosenquist, T.A. (2003) Germline transmission of RNAi in mice. *Nature Structural Biology*, **10**, 91-92.

- Carpenter, L. and Zernicka-Goetz, M. (2004) Directing pluripotent cell differentiation using "diced RNA" in transient transfection. *Genesis*, **40**, 157-163.
- Cartwright, P., McLean, C., Sheppard, A., Rivett, D., Jones, K. and Dalton, S. (2005) LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development*, **132**, 885-896.
- Caudy, A.A., Myers, M., Hannon, G.J. and Hammond, S.M. (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes & Development*, **16**, 2491-2496.
- Cerutti, L., Mian, N. and Bateman, A. (2000) Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. *Trends Biochem Sci*, **25**, 481-482.
- Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. and Smith, A. (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*, **113**, 643-655.
- Chambers, I. and Smith, A. (2004) Self-renewal of teratocarcinoma and embryonic stem cells. *Oncogene*, **23**, 7150-7160.
- Colognato, H. and Yurchenco, P.D. (2000) Form and function: the laminin family of heterotrimers. *Dev Dyn*, **218**, 213-234.
- Connelly, J.C., Kirkham, L.A. and Leach, D.R.F. (1998) The SbcCD nuclease of *Escherichia coli* is a structural maintenance of chromosomes (SMC) family protein that cleaves hairpin DNA. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 7969-7974.
- Cougot, N., Babajko, S. and Seraphin, B. (2004) Cytoplasmic foci are sites of mRNA decay in human cells. *J Cell Biol*, **165**, 31-40.
- Coumoul, X., Li, W., Wang, R.H. and Deng, C. (2004) Inducible suppression of Fgfr2 and Survivin in ES cells using a combination of the RNA interference (RNAi) and the Cre-LoxP system. *Nucleic Acids Res*, **32**, e85.
- Cserjesi, P., Brown, D., Lyons, G.E. and Olson, E.N. (1995) Expression of the novel basic helix-loop-helix gene eHAND in neural crest derivatives and extraembryonic membranes during mouse development. *Dev Biol*, **170**, 664-678.
- Cullen, L.M. and Arndt, G.M. (2005) Genome-wide screening for gene function using RNAi in mammalian cells. *Immunol Cell Biol*, **83**, 217-223.
- Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F. and Hannon, G.J. (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature*, **432**, 231-235.

- Denti, M.A., Rosa, A., Sthandier, O., De Angelis, F.G. and Bozzoni, I. (2004) A new vector, based on the PolII promoter of the U1 snRNA gene, for the expression of siRNAs in mammalian cells. *Mol Ther*, **10**, 191-199.
- Desbaillets, I., Ziegler, U., Groscurth, P. and Gassmann, M. (2000) Embryoid bodies: An in vitro model of mouse embryogenesis. *Experimental Physiology*, **85**, 645-651.
- Devroe, E. and Silver, P.A. (2002) Retrovirus-delivered siRNA. *BMC Biotechnology*, **2**, 15.
- Ding, Y., Chan, C.Y. and Lawrence, C.E. (2004) Sfold web server for statistical folding and rational design of nucleic acids. *Nucleic Acids Res*, **32**, W135-141.
- Ding, Y. and Lawrence, C.E. (2001) Statistical prediction of single-stranded regions in RNA secondary structure and application to predicting effective antisense target sites and beyond. *Nucleic Acids Res*, **29**, 1034-1046.
- Ding, Y. and Lawrence, C.E. (2003) A statistical sampling algorithm for RNA secondary structure prediction. *Nucleic Acids Res*, **31**, 7280-7301.
- Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K. and Saigo, K. (2003) Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. *Current Biology*, **13**, 41-46.
- Donze, O. and Picard, D. (2002) RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. *Nucleic Acids Research*, **30**, e46.
- Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature (London)*, **411**, 494-498.
- Elbashir, S.M., Harborth, J., Weber, K. and Tuschl, T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods (Orlando)*, **26**, 199-213.
- Elbashir, S.M., Lendeckel, W. and Tuschl, T. (2001b) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes & Development*, **15**, 188-200.
- Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W. and Tuschl, T. (2001c) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO (European Molecular Biology Organization) Journal*, **20**, 6877-6888.
- Era, T. and Witte, O.N. (2000) Regulated expression of P210 Bcr-Abl during embryonic stem cell differentiation stimulates multipotential progenitor expansion and myeloid cell fate. *Proc Natl Acad Sci U S A*, **97**, 1737-1742.
- Evans, M.J. and Kaufman, M.J. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature*, **292**, 154-156.

- Feil, R., Wagner, J., Metzger, D. and Chambon, P. (1997) Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem Biophys Res Commun*, **237**, 752-757.
- Filippov, V., Solovyev, V., Filippova, M. and Gill, S.S. (2000) A novel type of RNase III family proteins in eukaryotes. *Gene*, **245**, 213-221.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391**, 806-811.
- Forstemann, K., Tomari, Y., Du, T., Vagin, V.V., Denli, A.M., Bratu, D.P., Klattenhoff, C., Theurkauf, W.E. and Zamore, P.D. (2005) Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol*, **3**, e236.
- Fortin, K.R., Nicholson, R.H. and Nicholson, A.W. (2002) Mouse ribonuclease III. cDNA structure, expression analysis, and chromosomal location. *BMC Genomics*, **3**, 26.
- Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M. and Ahringer, J. (2000) Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature (London)*, **408**, 325-330.
- Gitlin, L., Karelsky, S. and Andino, R. (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature (London)*, **418**, 430-434.
- Gossen, M. and Bujard, H. (1995) Tight control of gene expression in eucaryotic cells by tetracycline-responsive promoters. *Official Gazette of the United States Patent and Trademark Office Patents*, **1180**, 327.
- Gossen, M., Freundlieb, S., Bender, G., Mueller, G., Hillen, W. and Bujard, H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science*, **268**, 1766-1769.
- Grabarek, J.B., Wianny, F., Plusa, B., Zernicka-Goetz, M. and Glover, D.M. (2003) RNA interference by production of short hairpin dsRNA in ES cells, their differentiated derivatives, and in somatic cell lines. *Biotechniques*, **34**, 734-736, 739-744.
- Gregory, R.I., Yan, K.P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. and Shiekhattar, R. (2004) The Microprocessor complex mediates the genesis of microRNAs. *Nature*, **432**, 235-240.
- Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G. and Mello, C.C. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell*, **106**, 23-34.

- Grishok, A., Tabara, H. and Mello, C.C. (2000) Genetic requirements for inheritance of RNAi in *C. elegans*. *Science*, **287**, 2494-2497.
- Ha, I., Wightman, B. and Ruvkun, G. (1996) A bulged lin-4/lin-14 RNA duplex is sufficient for *Caenorhabditis elegans* lin-14 temporal gradient formation. *Genes & Development*, **10**, 3041-3050.
- Haley, B. and Zamore, P.D. (2004) Kinetic analysis of the RNAi enzyme complex. *Nat Struct Mol Biol*, **11**, 599-606.
- Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature*, **404**, 293-296.
- Han, J., Lee, Y., Yeom, K.H., Kim, Y.K., Jin, H. and Kim, V.N. (2004) The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev*, **18**, 3016-3027.
- Harborth, J., Elbashir, S.M., Bechert, K., Tuschl, T. and Weber, K. (2001) Identification of essential genes in cultured mammalian cells using small interfering RNAs. *Journal of Cell Science*, **114**, 4557-4565.
- Hasuwa, H., Kaseda, K., Einarsdottir, T. and Okabe, M. (2002) Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Letters*, **532**, 227-230.
- Hay, D.C., Sutherland, L., Clark, J. and Burdon, T. (2004) Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. *Stem Cells*, **22**, 225-235.
- He, L. and Hannon, G.J. (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*, **5**, 522-531.
- Hemann, M.T., Fridman, J.S., Zilfou, J.T., Hernando, E., Paddison, P.J., Cordon-Cardo, C., Hannon, G.J. and Lowe, S.W. (2003) An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo. *Nature Genetics*, **33**, 396-400.
- Henkemeyer, M., Rossi, D.J., Holmyard, D.P., Puri, M.C., Mbamalu, G., Harpal, K., Shih, T.S., Jacks, T. and Pawson, T. (1995) Vascular system defects and neuronal apoptosis in mice lacking ras GTPase-activating protein. *Nature*, **377**, 695-701.
- Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E. and Prydz, H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. *Nucleic Acids Research*, **30**, 1757-1766.
- Houbaviy, H.B., Murray, M.F. and Sharp, P.A. (2003) Embryonic Stem Cell-Specific MicroRNAs. *Dev Cell*, **5**, 351-358.

- Houdebine, L.-M. (2002) The methods to generate transgenic animals and to control transgene expression. *Journal of Biotechnology*, **98**, 145-160.
- Humphrey, R.K., Beattie, G.M., Lopez, A.D., Bucay, N., King, C.C., Firpo, M.T., Rose-John, S. and Hayek, A. (2004) Maintenance of Pluripotency in Human Embryonic Stem Cells Is STAT3 Independent. *Stem Cells*, **22**, 522-530.
- Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T. and Zamore, P.D. (2001) A cellular function for the RNA-interference enzyme dicer in the maturation of the let-7 small temporal RNA. *Science (Washington D C)*, **293**, 834-838.
- Hutvagner, G. and Zamore, P.D. (2002) RNAi: nature abhors a double-strand. *Curr Opin Genet Dev*, **12**, 225-232.
- Ishizuka, A., Siomi, M.C. and Siomi, H. (2002) A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev*, **16**, 2497-2508.
- Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P.S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnology*, **21**, 635-637.
- Kamath, R.S. and Ahringer, J. (2003) Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods*, **30**, 313-321.
- Kamath, R.S., Martinez-Campos, M., Zipperlen, P., Fraser, A.G. and Ahringer, J. (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol*, **2**, RESEARCH0002.
- Kanellopoulou, C., Muljo, S.A., Kung, A.L., Ganesan, S., Drapkin, R., Jenuwein, T., Livingston, D.M. and Rajewsky, K. (2005) Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev*, **19**, 489-501.
- Kawasaki, H., Suyama, E., Iyo, M. and Taira, K. (2003) siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells. *Nucleic Acids Research*, **31**, 981-987.
- Keller, G.M. (1995) *In vitro* differentiation of embryonic stem cells. *Current Opinion in Cell Biology*, **7**, 862-869.
- Kennerdell, J.R. and Carthew, R.W. (2000) Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nature Biotechnology*, **18**, 896-898.

- Kennerdell, J.R., Yamaguchi, S. and Carthew, R.W. (2002) RNAi is activated during *Drosophila* oocyte maturation in a manner dependent on aubergine and spindle-E. *Genes Dev*, **16**, 1884-1889.
- Ketting, R.F., Fischer, S.E.J., Bernstein, E., Sijen, T., Hannon, G.J. and Plasterk, R.H.A. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes & Development*, **15**, 2654-2659.
- Ketting, R.F. and Plasterk, R.H.A. (2000) A genetic link between co-suppression and RNA interference in *C. elegans*. *Nature*, **404**, 296-298.
- Khvorova, A., Reynolds, A. and Jayasena, S.D. (2003) Functional siRNAs and miRNAs Exhibit Strand Bias. *Cell*, **115**, 209-216.
- Knight, S.W. and Bass, B.L. (2001) A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. *Science (Washington D C)*, **293**, 2269-2271.
- Koper-Emde, D., Herrmann, L., Sandrock, B. and Benecke, B.J. (2004) RNA interference by small hairpin RNAs synthesised under control of the human 7S K RNA promoter. *Biol Chem*, **385**, 791-794.
- Kunath, T., Arnaud, D., Uy, G.D., Okamoto, I., Chureau, C., Yamanaka, Y., Heard, E., Gardner, R.L., Avner, P. and Rossant, J. (2005) Imprinted X-inactivation in extra-embryonic endoderm cell lines from mouse blastocysts. *Development*, **132**, 1649-1661.
- Kunath, T., Gish, G., Lickert, H., Jones, N., Pawson, T. and Rossant, J. (2003) Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. *Nature Biotechnology*, **21**, 559-561.
- 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., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J.P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J.C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R.H., Wilson, R.K., Hillier, L.W., McPherson, J.D., Marra, M.A., Mardis, E.R., Fulton, L.A., Chinwalla, A.T., Pepin, K.H., Gish, W.R., Chissoe, S.L., Wendl, M.C., Delehaunty, K.D., Miner, T.L., Delehaunty, A., Kramer, J.B., Cook, L.L., Fulton, R.S., Johnson, D.L., Minx, P.J., Clifton, S.W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J.F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R.A., Muzny, D.M., Scherer, S.E.,

- Bouck, J.B., Sodergren, E.J., Worley, K.C., Rives, C.M., Gorrell, J.H., Metzker, M.L., Naylor, S.L., Kucherlapati, R.S., Nelson, D.L., Weinstock, G.M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Smith, D.R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H.M., Dubois, J., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R.W., Federspiel, N.A., Abola, A.P., Proctor, M.J., Myers, R.M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D.R., Olson, M.V., Kaul, R., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G.A., Athanasiou, M., Schultz, R., Roe, B.A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W.R., de la Bastide, M., Dedhia, N., Blocker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J.A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D.G., Burge, C.B., Cerutti, L., Chen, H.C., Church, D., Clamp, M., Copley, R.R., Doerks, T., Eddy, S.R., Eichler, E.E., Furey, T.S., Galagan, J., Gilbert, J.G., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L.S., Jones, T.A., Kasif, S., Kasprzyk, A., Kennedy, S., Kent, W.J., Kitts, P., Koonin, E.V., Korf, I., Kulp, D., Lancet, D., Lowe, T.M., McLysaght, A., Mikkelsen, T., Moran, J.V., Mulder, N., Pollara, V.J., Ponting, C.P., Schuler, G., Schultz, J., Slater, G., Smit, A.F., Stupka, E., Szustakowski, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y.I., Wolfe, K.H., Yang, S.P., Yeh, R.F., Collins, F., Guyer, M.S., Peterson, J., Felsenfeld, A., Wetterstrand, K.A., Patrinos, A., Morgan, M.J., Szustakowski, J., de Jong, P., Catanese, J.J., Osoegawa, K., Shizuya, H., Choi, S. and Chen, Y.J. (2001) Initial sequencing and analysis of the human genome. *Nature*, **409**, 860-921.
- Lebedeva, I. and Stein, C.A. (2001) Antisense oligonucleotides: Promise and reality. *Annual Review of Pharmacology and Toxicology*, **41**, 403-419.
- Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. and Kim, V.N. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature*, **425**, 415-419.
- Lee, Y., Jeon, K., Lee, J.-T., Kim, S. and Kim, V.N. (2002) MicroRNA maturation: Stepwise processing and subcellular localization. *EMBO (European Molecular Biology Organization) Journal*, **21**, 4663-4670.
- Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J. and Carthew, R.W. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*, **117**, 69-81.
- Leirdal, M. and Sioud, M. (2002) Gene silencing in mammalian cells by preformed small RNA duplexes. *Biochemical and Biophysical Research Communications*, **295**, 744-748.
- Lette, G., Kritikou, E.A., Jaeggi, M., Calixto, A., Fraser, A.G., Kamath, R.S., Ahringer, J. and Hengartner, M.O. (2004) Genome-wide RNAi identifies p53-

- dependent and -independent regulators of germ cell apoptosis in *C. elegans*. *Cell Death Differ*, **11**, 1198-1203.
- Li, E., Bestor, T.H. and Jaenisch, R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, **69**, 915-926.
- Lim, L.P., Glasner, M.E., Yekta, S., Burge, C.B. and Bartel, D.P. (2003a) Vertebrate microRNA genes. *Science*, **299**, 1540.
- Lim, L.P., Lau, N.C., Weinstein, E.G., Abdelhakim, A., Yekta, S., Rhoades, M.W., Burge, C.B. and Bartel, D.P. (2003b) The microRNAs of *Caenorhabditis elegans*. *Genes & Development*, **17**, 991-1008.
- Lin, T., Chao, C., Saito, S., Mazur, S.J., Murphy, M.E., Appella, E. and Xu, Y. (2005) p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol*, **7**, 165-171.
- Lingel, A., Simon, B., Izaurralde, E. and Sattler, M. (2004) Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. *Nat Struct Mol Biol*, **11**, 576-577.
- Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L. and Hannon, G.J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. *Science*, **305**, 1437-1441.
- Liu, Q., Rand, T.A., Kalidas, S., Du, F., Kim, H.E., Smith, D.P. and Wang, X. (2003) R2D2, a Bridge Between the Initiation and Effector Steps of the *Drosophila* RNAi Pathway. *Science*, **301**, 1921-1925.
- Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R. and Golub, T.R. (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834-838.
- Lund, E., Guttinger, S., Calado, A., Dahlberg, J.E. and Kutay, U. (2004) Nuclear export of microRNA precursors. *Science*, **303**, 95-98.
- Maeda, I., Kohara, Y., Yamamoto, M. and Sugimoto, A. (2001) Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. *Curr Biol*, **11**, 171-176.
- Manche, L., Green, S.R., Schmedt, C. and Mathews, M.B. (1992) Interaction between double-stranded RNA regulators and the protein kinase DAI. *Molecular and Cellular Biology*, **12**, 5238-5248.
- Martin, G.R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A*, **78**, 7634-7638.

- Martinez, J., Patkaniowska, A., Urlaub, H., Luehrmann, R. and Tuschl, T. (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell*, **110**, 563-574.
- Martinez, J. and Tuschl, T. (2004) RISC is a 5' phosphomonoester-producing RNA endonuclease. *Genes Dev.*
- Matin, M.M., Walsh, J.R., Gokhale, P.J., Draper, J.S., Bahrami, A.R., Morton, I., Moore, H.D. and Andrews, P.W. (2004) Specific Knockdown of Oct4 and β 2-microglobulin Expression by RNA Interference in Human Embryonic Stem Cells and Embryonic Carcinoma Cells. *Stem Cells*, **22**, 659-668.
- McCaffrey, A.P., Meuse, L., Pham, T.-T.T., Conklin, D.S., Hannon, G.J. and Kay, M.A. (2002) RNA interference in adult mice. *Nature*, **418**, 38-39.
- McCaffrey, A.P., Nakai, H., Pandey, K., Huang, Z., Salazar, F.H., Hui, X., Wieland, S., Marion, P.L. and Kay, M.A. (2003) Inhibition of hepatitis B virus in mice by RNA interference. *Nature Biotechnology*, **21**, 639-644.
- McManus, M.T., Haines, B.B., Dillon, C.P., Whitehurst, C.E., Van Parijs, L., Chen, J. and Sharp, P.A. (2002) Small interfering RNA-mediated gene silencing in T lymphocytes. *Journal of Immunology*, **169**, 5754-5760.
- Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G. and Tuschl, T. (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell*, **15**, 185-197.
- Miner, J.H., Li, C., Mudd, J.L., Go, G. and Sutherland, A.E. (2004) Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation. *Development*, **131**, 2247-2256.
- Misquitta, L. and Paterson, B.M. (1999) Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): A role for nautilus in embryonic somatic muscle formation. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 1451-1456.
- Mitchell, K.J., Pinson, K.I., Kelly, O.G., Brennan, J., Zupicich, J., Scherz, P., Leighton, P.A., Goodrich, L.V., Lu, X., Avery, B.J., Tate, P., Dill, K., Pangilinan, E., Wakenight, P., Tessier-Lavigne, M. and Skarnes, W.C. (2001) Functional analysis of secreted and transmembrane proteins critical to mouse development. *Nat Genet*, **28**, 241-249.
- Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M. and Yamanaka, S. (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*, **113**, 631-642.
- Miyagishi, M. and Taira, K. (2002) U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nature Biotechnology*, **20**, 497-500.

- Morimoto, H., Okamura, H., Yoshida, K., Kitamura, S. and Haneji, T. (2004) Double-stranded RNA mediates selective gene silencing of protein phosphatase type 1 delta isoform in HEK-293 cells. *J Enzyme Inhib Med Chem*, **19**, 327-331.
- Moutier, R., Tchang, F., Caucheteux, S.M. and Kanellopoulos-Langevin, C. (2003) Placental anomalies and fetal loss in mice, after administration of doxycycline in food for tet-system activation. *Transgenic Res*, **12**, 369-373.
- Myers, J.W., Jones, J.T., Meyer, T. and Ferrell, J.E., Jr. (2003) Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. *Nature Biotechnology*, **21**, 324-328.
- Napoli, C., Lemieux, C. and Jorgensen, R. (1990) Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell*, **2**, 279-289.
- Nichols, J., Evans, E.P. and Smith, A.G. (1990) Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity. *Development*, **110**, 1341-1348.
- Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schoeler, H. and Smith, A. (1998) Formation and pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*, **95**, 379-391.
- Nishimoto, M., Fukushima, A., Okuda, A. and Muramatsu, M. (1999) The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. *Mol Cell Biol*, **19**, 5453-5465.
- Niwa, H., Burdon, T., Chambers, I. and Smith, A. (1998) Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev*, **12**, 2048-2060.
- Niwa, H., Miyazaki, J.-i. and Smith, A.G. (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genetics*, **24**, 372-376.
- Nykanen, A., Haley, B. and Zamore, P.D. (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell*, **107**, 309-321.
- Okamura, K., Ishizuka, A., Siomi, H. and Siomi, M.C. (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev*.
- Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. and Conklin, D.S. (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes & Development*, **16**, 948-958.

- Paddison, P.J., Silva, J.M., Conklin, D.S., Schlabach, M., Li, M., Aruleba, S., Balija, V., O'Shaughnessy, A., Gnoj, L., Scobie, K., Chang, K., Westbrook, T., Cleary, M., Sachidanandam, R., McCombie, W.R., Elledge, S.J. and Hannon, G.J. (2004) A resource for large-scale RNA-interference-based screens in mammals. *Nature*, **428**, 427-431.
- Paling, N.R., Wheadon, H., Bone, H.K. and Welham, M.J. (2004) Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. *J Biol Chem*, **279**, 48063-48070.
- 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. *Developmental Biology*, **166**, 259-267.
- Parker, J.S., Roe, S.M. and Barford, D. (2004) Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. *Embo J*, **23**, 4727-4737.
- Paul, C.P., Good, P.D., Winer, I. and Engelke, D.R. (2002) Effective expression of small interfering RNA in human cells. *Nature Biotechnology*, **20**, 505-508.
- Piccin, A., Salameh, A., Benna, C., Sandrelli, F., Mazzotta, G., Zordan, M., Rosato, E., Kyriacou, C.P. and Costa, R. (2001) Efficient and heritable functional knock-out of an adult phenotype in *Drosophila* using a GAL4-driven hairpin RNA incorporating a heterologous spacer. *Nucleic Acids Res*, **29**, E55-55.
- Plasterk, R.H. (2002) RNA silencing: the genome's immune system. *Science*, **296**, 1263-1265.
- Pomerantz, R.J. (2002) RNA interference meets HIV-1: Will silence be golden? *Nature Medicine*, **8**, 659-660.
- Pothof, J., van Haften, G., Thijssen, K., Kamath, R.S., Fraser, A.G., Ahringer, J., Plasterk, R.H.A. and Tijsterman, M. (2003) Identification of genes that protect the *C. elegans* genome against mutations by genome-wide RNAi. *Genes & Development*, **17**, 443-448.
- Pratt, T., Sharp, L., Nichols, J., Price, D.J. and Mason, J.O. (2000) Embryonic stem cells and transgenic mice ubiquitously expressing a tau-tagged green fluorescent protein. *Dev Biol*, **228**, 19-28.
- Provost, P., Dishart, D., Doucet, J., Friendewey, D., Samuelsson, B. and Radmark, O. (2002) Ribonuclease activity and RNA binding of recombinant human Dicer. *EMBO (European Molecular Biology Organization) Journal*, **21**, 5864-5874.
- Qi, X., Li, T.G., Hao, J., Hu, J., Wang, J., Simmons, H., Miura, S., Mishina, Y. and Zhao, G.Q. (2004) BMP4 supports self-renewal of embryonic stem cells by

- inhibiting mitogen-activated protein kinase pathways. *Proc Natl Acad Sci U S A*, **101**, 6027-6032.
- Raff, T., van der Giet, M., Endemann, D., Wiederholt, T. and Paul, M. (1997) Design and testing of beta-actin primers for RT-PCR that do not co-amplify processed pseudogenes. *Biotechniques*, **23**, 456-460.
- Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W.S. and Khvorova, A. (2004) Rational siRNA design for RNA interference. *Nat Biotechnol*, **22**, 326-330.
- Rogers, M.B., Hosler, B.A. and Gudas, L.J. (1991) Specific Expression of a Retinoic Acid-Regulated Zinc-Finger Gene Rex-1 in Preimplantation Embryos Trophoblast and Spermatoocytes. *Development*, **113**, 815-824.
- Romano, N. and Macino, G. (1992) Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Mol Microbiol*, **6**, 3343-3353.
- Rosner, M.H., Vigano, M.A., Ozato, K., Timmons, P.M., Poirier, F., Rigby, P.W.J. and Staudt, L.M. (1990) A Pou-Domain Transcription Factor in Early Stem Cells and Germ Cells of the Mammalian Embryo. *Nature*, **345**, 686-692.
- Rubinson, D.A., Dillon, C.P., Kwiatkowski, A.V., Sievers, C., Yang, L., Kopinja, J., Zhang, M., McManus, M.T., Gertler, F.B., Scott, M.L. and Van Parijs, L. (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nature Genetics*, **33**, 401-406.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning - a Laboratory Manual*. New York, USA., Cold Spring Harbour.
- Sasaki, M., Kato, S., Kohno, K., Martin, G.R. and Yamada, Y. (1987) Sequence of the Complementary DNA Encoding the Laminin B1 Chain Reveals a Multidomain Protein Containing Cysteine-Rich Repeats. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 935-939.
- Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. and Brivanlou, A.H. (2004) Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med*, **10**, 55-63.
- Scherr, M., Morgan, M.A. and Eder, M. (2003) Gene silencing mediated by small interfering RNAs in mammalian cells. *Current Medicinal Chemistry*, **10**, 245-256.
- Scholer, H.R., Ruppert, S., Suzuki, N., Chowdhury, K. and Gruss, P. (1990) New Type of Pou Domain in Germ Line-Specific Protein Oct-4. *Nature*, **344**, 435-439.

- Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P.D. (2003) Asymmetry in the Assembly of the RNAi Enzyme Complex. *Cell*, **115**, 199-208.
- Schwarz, D.S., Hutvagner, G., Haley, B. and Zamore, P.D. (2002) Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. *Molecular Cell*, **10**, 537-548.
- Sen, G., Wehrman, T.S., Myers, J.W. and Blau, H.M. (2004) Restriction enzyme-generated siRNA (REGS) vectors and libraries. *Nat Genet*, **36**, 183-189.
- Sen, G.L. and Blau, H.M. (2005) Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol*.
- Shinagawa, T. and Ishii, S. (2003) Generation of Ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter. *Genes Dev*, **17**, 1340-1345.
- Siemering, K.R., Golbik, R., Sever, R. and Haseloff, J. (1996) Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr Biol*, **6**, 1653-1663.
- Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H.A. and Fire, A. (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell*, **107**, 465-476.
- Silva, J.M., Mizuno, H., Brady, A., Lucito, R. and Hannon, G.J. (2004) RNA interference microarrays: high-throughput loss-of-function genetics in mammalian cells. *Proc Natl Acad Sci U S A*, **101**, 6548-6552.
- Sledz, C.A., Holko, M., De Veer, M.J., Silverman, R.H. and Williams, B.R. (2003) Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol*, **5**, 834-839.
- Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., John, M., Kesavan, V., Lavine, G., Pandey, R.K., Racie, T., Rajeev, K.G., Rohl, I., Toudjarska, I., Wang, G., Wuschko, S., Bumcrot, D., Koteliansky, V., Limmer, S., Manoharan, M. and Vornlocher, H.P. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature*, **432**, 173-178.
- Stark, G.R., Kerr, I.M., Williams, B.R.G., Silverman, R.H. and Schreiber, R.D. (1998) How cells respond to interferons. *Annual Review of Biochemistry*, **67**, 227-264.
- Strathdee, C.A., McLeod, M.R. and Hall, J.R. (1999) Efficient control of tetracycline-responsive gene expression from an autoregulated bi-directional expression vector. *Gene*, **229**, 21-29.

- Sui, G., Soohoo, C., Affar, E.B., Gay, F., Shi, Y., Forrester, W.C. and Shi, Y. (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 5515-5520.
- Svoboda, P., Stein, P., Hayashi, H. and Schultz, R.M. (2000) Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development (Cambridge)*, **127**, 4147-4156.
- Svoboda, P., Stein, P. and Schultz, R.M. (2001) RNAi in mouse oocytes and preimplantation embryos: Effectiveness of hairpin dsRNA. *Biochemical and Biophysical Research Communications*, **287**, 1099-1104.
- Tabara, H., Grishok, A. and Mello, C.C. (1998) RNAi in *C. elegans*: soaking in the genome sequence. *Science*, **282**, 430-431.
- Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A. and Mello, C.C. (1999) The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell*, **99**, 123-132.
- Tabara, H., Yigit, E., Siomi, H. and Mello, C.C. (2002) The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in *C. elegans*. *Cell*, **109**, 861-871.
- Takada, T., Nemoto, K., Yamashita, A., Kato, M., Kondo, Y. and Torii, R. (2005) Efficient gene silencing and cell differentiation using siRNA in mouse and monkey ES cells. *Biochem Biophys Res Commun*, **331**, 1039-1044.
- Tanaka, S., Kunath, T., Hadjantonakis, A.-K., Nagy, A. and Rossant, J. (1998) Promotion of trophoblast stem cell proliferation by FGF4. *Science*, **282**, 2072-2075.
- Tang, F.C., Meng, G.L., Yang, H.B., Li, C.J., Shi, Y., Ding, M.X., Shang, K.G., Zhang, B. and Xue, Y.F. (2004) Stable suppression of gene expression in murine embryonic stem cells by RNAi directed from DNA vector-based short hairpin RNA. *Stem Cells*, **22**, 93-99.
- Tijsterman, M., Okihara, K.L., Thijssen, K. and Plasterk, R.H. (2002) PPW-1, a PAZ/PIWI protein required for efficient germline RNAi, is defective in a natural isolate of *C. elegans*. *Curr Biol*, **12**, 1535-1540.
- Timmons, L., Court, D.L. and Fire, A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene*, **263**, 103-112.
- Tiscornia, G., Singer, O., Ikawa, M. and Verma, I.M. (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 1844-1848.

- Tokuzawa, Y., Kaiho, E., Maruyama, M., Takahashi, K., Mitsui, K., Maeda, M., Niwa, H. and Yamanaka, S. (2003) Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. *Mol Cell Biol*, **23**, 2699-2708.
- Tomari, Y., Matranga, C., Haley, B., Martinez, N. and Zamore, P.D. (2004) A protein sensor for siRNA asymmetry. *Science*, **306**, 1377-1380.
- Tomari, Y. and Zamore, P.D. (2005) Perspective: machines for RNAi. *Genes Dev*, **19**, 517-529.
- Tomioka, M., Nishimoto, M., Miyagi, S., Katayanagi, T., Fukui, N., Niwa, H., Muramatsu, M. and Okuda, A. (2002) Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. *Nucleic Acids Res*, **30**, 3202-3213.
- Tuschl, T. (2002) Expanding small RNA interference. *Nature Biotechnology*, **20**, 446-448.
- Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R. and Saigo, K. (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res*, **32**, 936-948.
- Ui-Tei, K., Zenno, S., Miyata, Y. and Saigo, K. (2000) Sensitive assay of RNA interference in *Drosophila* and Chinese hamster cultured cells using firefly luciferase gene as target. *FEBS Lett*, **479**, 79-82.
- Urlinger, S., Baron, U., Thellmann, M., Hasan, M.T., Bujard, H. and Hillen, W. (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: Novel mutations yield expanded range and sensitivity. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 7963-7968.
- van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N. and Stuitje, A.R. (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell*, **2**, 291-299.
- Velkey, J.M. and O'Shea, K.S. (2003) Oct4 RNA interference induces trophectoderm differentiation in mouse embryonic stem cells. *Genesis*, **37**, 18-24.
- Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A., Gocayne, J.D., Amanatides, P., Ballew, R.M., Huson, D.H., Wortman, J.R., Zhang, Q., Kodira, C.D., Zheng, X.H., Chen, L., Skupski, M., Subramanian, G., Thomas, P.D., Zhang, J., Gabor Miklos, G.L., Nelson, C., Broder, S., Clark, A.G., Nadeau, J., McKusick, V.A., Zinder, N., Levine, A.J., Roberts, R.J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K.,

- Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di Francesco, V., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A.E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T.J., Higgins, M.E., Ji, R.R., Ke, Z., Ketchum, K.A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G.V., Milshina, N., Moore, H.M., Naik, A.K., Narayan, V.A., Neelam, B., Nusskern, D., Rusch, D.B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M.L., Curry, L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D., McCawley, S., McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y.H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N.N., Tse, S., Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J.F., Guigo, R., Campbell, M.J., Sjolander, K.V., Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T., Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y.H., Coyne, M., Dahlke, C., Mays, A., Dombroski, M., Donnelly, M., Ely, D., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., Newman, M., Nguyen, T., Nguyen, N., Nodell, M., Pan, S., Peck, J., Peterson, M., Rowe, W., Sanders, R., Scott, J., Simpson, M., Smith, T., Sprague, A., Stockwell, T., Turner, R., Venter, E., Wang, M., Wen, M., Wu, D., Wu, M., Xia, A., Zandieh, A. and Zhu, X. (2001) The sequence of the human genome. *Science*, **291**, 1304-1351.
- Ventura, A., Meissner, A., Dillon, C.P., McManus, M., Sharp, P.A., Van Parijs, L., Jaenisch, R. and Jacks, T. (2004) Cre-lox-regulated conditional RNA interference from transgenes. *Proc Natl Acad Sci U S A*, **101**, 10380-10385.
- Voinnet, O., Vain, P., Angell, S. and Baulcombe, D.C. (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell*, **95**, 177-187.
- Wang, J., Tekle, E., Oubrahim, H., Mieyal, J.J., Stadtman, E.R. and Chock, P.B. (2003) Stable and controllable RNA interference: Investigating the physiological function of glutathionylated actin. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 5103-5106.

- Wang, Y.H., Liu, S., Zhang, G., Zhou, C.Q., Zhu, H.X., Zhou, X.B., Quan, L.P., Bai, J.F. and Xu, N.Z. (2005) Knockdown of c-Myc expression by RNAi inhibits MCF-7 breast tumor cells growth in vitro and in vivo. *Breast Cancer Res*, **7**, R220-228.
- Waterhouse, P.M., Graham, M.W. and Wang, M.B. (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc Natl Acad Sci U S A*, **95**, 13959-13964.
- Waterston, R.H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J.F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P., Antonarakis, S.E., Attwood, J., Baertsch, R., Bailey, J., Barlow, K., Beck, S., Berry, E., Birren, B., Bloom, T., Bork, P., Botcherby, M., Bray, N., Brent, M.R., Brown, D.G., Brown, S.D., Bult, C., Burton, J., Butler, J., Campbell, R.D., Carninci, P., Cawley, S., Chiaromonte, F., Chinwalla, A.T., Church, D.M., Clamp, M., Clee, C., Collins, F.S., Cook, L.L., Copley, R.R., Coulson, A., Couronne, O., Cuff, J., Curwen, V., Cutts, T., Daly, M., David, R., Davies, J., Delehaunty, K.D., Deri, J., Dermitzakis, E.T., Dewey, C., Dickens, N.J., Diekhans, M., Dodge, S., Dubchak, I., Dunn, D.M., Eddy, S.R., Elnitski, L., Emes, R.D., Eswara, P., Eyraas, E., Felsenfeld, A., Fewell, G.A., Flicek, P., Foley, K., Frankel, W.N., Fulton, L.A., Fulton, R.S., Furey, T.S., Gage, D., Gibbs, R.A., Glusman, G., Gnerre, S., Goldman, N., Goodstadt, L., Grafham, D., Graves, T.A., Green, E.D., Gregory, S., Guigo, R., Guyer, M., Hardison, R.C., Haussler, D., Hayashizaki, Y., Hillier, L.W., Hinrichs, A., Hlavina, W., Holzer, T., Hsu, F., Hua, A., Hubbard, T., Hunt, A., Jackson, I., Jaffe, D.B., Johnson, L.S., Jones, M., Jones, T.A., Joy, A., Kamal, M., Karlsson, E.K., Karolchik, D., Kasprzyk, A., Kawai, J., Keibler, E., Kells, C., Kent, W.J., Kirby, A., Kolbe, D.L., Korf, I., Kucherlapati, R.S., Kulbokas, E.J., Kulp, D., Landers, T., Leger, J.P., Leonard, S., Letunic, I., Levine, R., Li, J., Li, M., Lloyd, C., Lucas, S., Ma, B., Maglott, D.R., Mardis, E.R., Matthews, L., Mauceli, E., Mayer, J.H., McCarthy, M., McCombie, W.R., McLaren, S., McLay, K., McPherson, J.D., Meldrim, J., Meredith, B., Mesirov, J.P., Miller, W., Miner, T.L., Mongin, E., Montgomery, K.T., Morgan, M., Mott, R., Mullikin, J.C., Muzny, D.M., Nash, W.E., Nelson, J.O., Nhan, M.N., Nicol, R., Ning, Z., Nusbaum, C., O'Connor, M.J., Okazaki, Y., Oliver, K., Overton-Larty, E., Pachter, L., Parra, G., Pepin, K.H., Peterson, J., Pevzner, P., Plumb, R., Pohl, C.S., Poliakov, A., Ponce, T.C., Ponting, C.P., Potter, S., Quail, M., Reymond, A., Roe, B.A., Roskin, K.M., Rubin, E.M., Rust, A.G., Santos, R., Sapojnikov, V., Schultz, B., Schultz, J., Schwartz, M.S., Schwartz, S., Scott, C., Seaman, S., Searle, S., Sharpe, T., Sheridan, A., Shownkeen, R., Sims, S., Singer, J.B., Slater, G., Smit, A., Smith, D.R., Spencer, B., Stabenau, A., Stange-Thomann, N., Sugnet, C., Suyama, M., Tesler, G., Thompson, J., Torrents, D., Trevaskis, E., Tromp, J., Ucla, C., Ureta-Vidal, A., Vinson, J.P., Von Niederhausern, A.C., Wade, C.M., Wall, M., Weber, R.J., Weiss, R.B., Wendl, M.C., West, A.P., Wetterstrand, K., Wheeler, R., Whelan, S., Wierzbowski, J., Willey, D., Williams, S., Wilson, R.K., Winter, E., Worley, K.C., Wyman, D., Yang, S., Yang, S.P., Zdobnov, E.M., Zody, M.C. and Lander, E.S. (2002) Initial sequencing and comparative analysis of the mouse genome. *Nature*, **420**, 520-562.

- Wianny, F. and Zernicka-Goetz, M. (2000) Specific interference with gene function by double-stranded RNA in early mouse development. *Nature Cell Biology*, **2**, 70-75.
- Williams, N.S., Gaynor, R.B., Scoggin, S., Verma, U., Gokaslan, T., Simmang, C., Fleming, J., Tavana, D., Frenkel, E. and Becerra, C. (2003) Identification and validation of genes involved in the pathogenesis of colorectal cancer using cDNA microarrays and RNA interference. *Clinical Cancer Research*, **9**, 931-946.
- Yague, E., Higgins, C.F. and Raguz, S. (2004) Complete reversal of multidrug resistance by stable expression of small interfering RNAs targeting MDR1. *Gene Ther.*
- Yang, D., Buchholz, F., Huang, Z., Goga, A., Chen, C.-Y., Brodsky, F.M. and Bishop, J.M. (2002) Short RNA duplexes produced by hydrolysis with *Escherichia coli* RNase III mediate effective RNA interference in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 9942-9947.
- Yang, S., Tutton, S., Pierce, E. and Yoon, K. (2001) Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. *Molecular and Cellular Biology*, **21**, 7807-7816.
- Yekta, S., Shih, I.H. and Bartel, D.P. (2004) MicroRNA-directed cleavage of HOXB8 mRNA. *Science*, **304**, 594-596.
- Ying, Q.L., Nichols, J., Chambers, I. and Smith, A. (2003) BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell*, **115**, 281-292.
- Yokota, T., Sakamoto, N., Enomoto, N., Tanabe, Y., Miyagishi, M., Maekawa, S., Yi, L., Kurosaki, M., Taira, K., Watanabe, M. and Mizusawa, H. (2003) Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep*, **4**, 602-608.
- Yu, J.-Y., DeRuiter, S.L. and Turner, D.L. (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 6047-6052.
- Yuan, H., Corbi, N., Basilico, C. and Dailey, L. (1995) Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. *Genes Dev*, **9**, 2635-2645.
- Zaehres, H., Lensch, M.W., Daheron, L., Stewart, S.A., Itskovitz-Eldor, J. and Daley, G.Q. (2005) High-Efficiency RNA Interference in Human Embryonic Stem Cells. *Stem Cells*, **23**, 299-305.

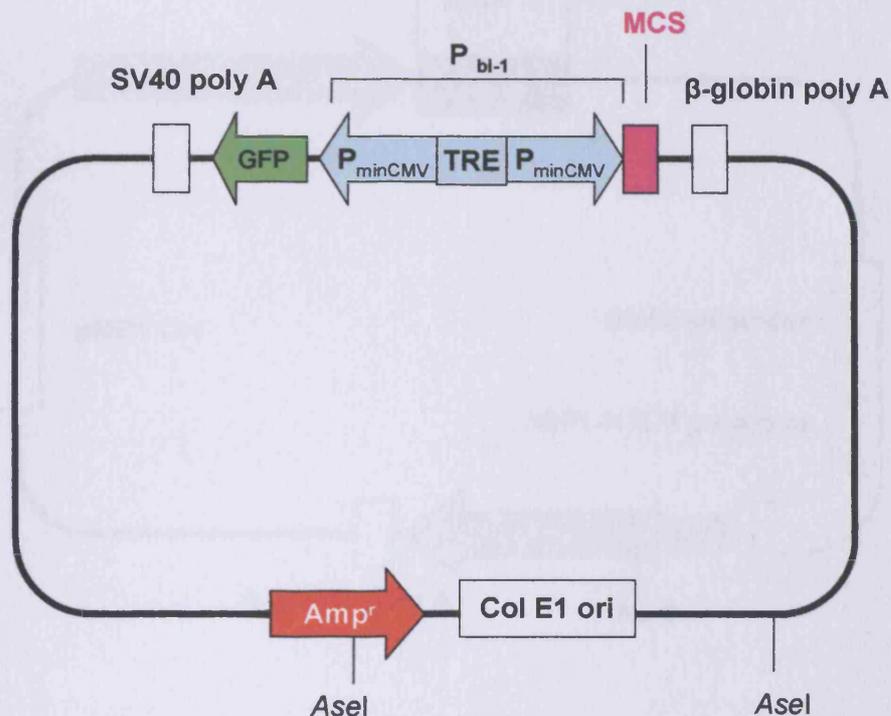
- Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000) RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, **101**, 25-33.
- Zeng, Y. and Cullen, B.R. (2002) RNA interference in human cells is restricted to the cytoplasm. *Rna*, **8**, 855-860.
- Zernicka-Goetz, M., Pines, J., Hunter, S.M., Dixon, J.P.C., Siemering, K.R., Haseloff, J. and Evans, M.J. (1997) Following cell fate in the living mouse embryo. *Development*, **124**, 1133-1137.
- Zhang, H., Fabrice, K., A., Brondani, V., Billy, E. and Witold, F. (2002) Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *EMBO (European Molecular Biology Organization) Journal*, **21**, 5875-5885.
- Zhao, G.Q. (2003) Consequences of knocking out BMP signaling in the mouse. *Genesis*, **35**, 43-56.

Appendix 1

Vector Maps

Appendix 1: Vector Maps

A1.1. pBI-EGFP



Modified MCS

600 *SpeI* *ClaI*
 AGTCAGCTGACGCGTCGGGACTAGTCCCGATCGATGGACCATT

645 *XhoI* *BstBI* *NheI*
 GCACTCGAGCGGACTTCGAAGCATGCGCGTGCTAGCGCGCCCT

Figure A1.1 pBI-EGFP (BD Biosciences, Oxford, UK). This vector was used for cloning the inverted repeats of part of the *Oct-4* and *Lamin A/C* cDNA (Chapter 5). The MCS site was modified for ease of cloning. The plasmid has a bidirectional promoter that has the tetracycline response element (TRE) positioned between two identical minimal CMV promoters (P_{minCMV}). One promoter controls the expression of green fluorescence protein (GFP) and the second promoter controls the expression of hairpin dsRNA (hpRNA) that had been cloned in. Bacteria containing the vector can be selected for using ampicillin. The vector was digested with *AseI* restriction enzymes and then gel purified before transfection into cells.

A1.2. psiRNA-hH1zeo

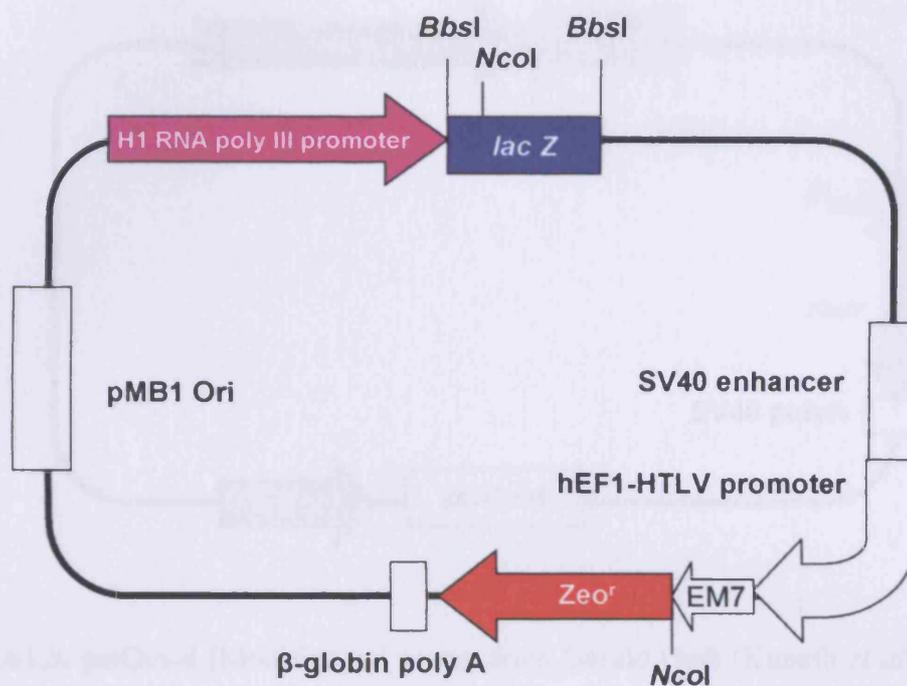


Figure A1.2. psiRNA-hH1zeo (Autogen Bioclear UK Ltd., Wiltshire, UK.). This vector was used for expression of shRNA targeting *Laminin B1* and *Rex-1* (Chapter 4). Annealed oligos are cloned into the *BbsI* sites of the plasmid. Insertion of the oligos replaces *lacZ* allowing identification of bacteria transformed with vector containing the insert. Presence of the insert was confirmed by *NcoI* restriction digest. Expression of *Sh ble* confers resistance to the antibiotic Zeocin™ allowing selection of transfected cells containing the vector. The shRNA is transcribed from the H1 RNA polymerase III promoter.

A1.3. psiOct-4

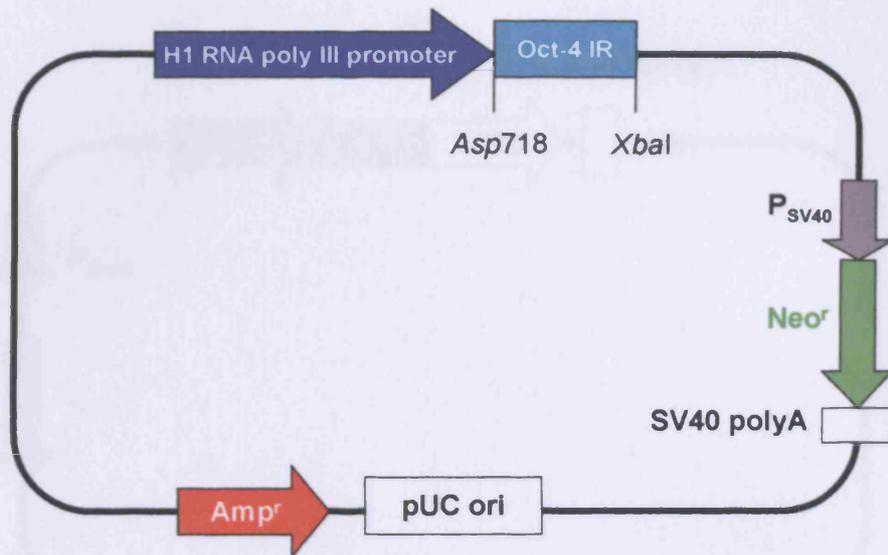


Figure A1.3. psiOct-4 [Modified psi vector from Gerald Gish (Kunath *et al.*, 2003). A derivative of this vector (psiOct4) was made (by Lars Grotewold, Edinburgh University, UK).] Expression of the shRNA targeting oct-4 is under the control of the Human H1 RNA polymerase III promoter. The vector also has neomycin resistance for selection of stably transfected cell lines. *Asp718* and *XbaI* sites can be used for cloning of other inverted repeat sequences.

A1.4. pTet-On

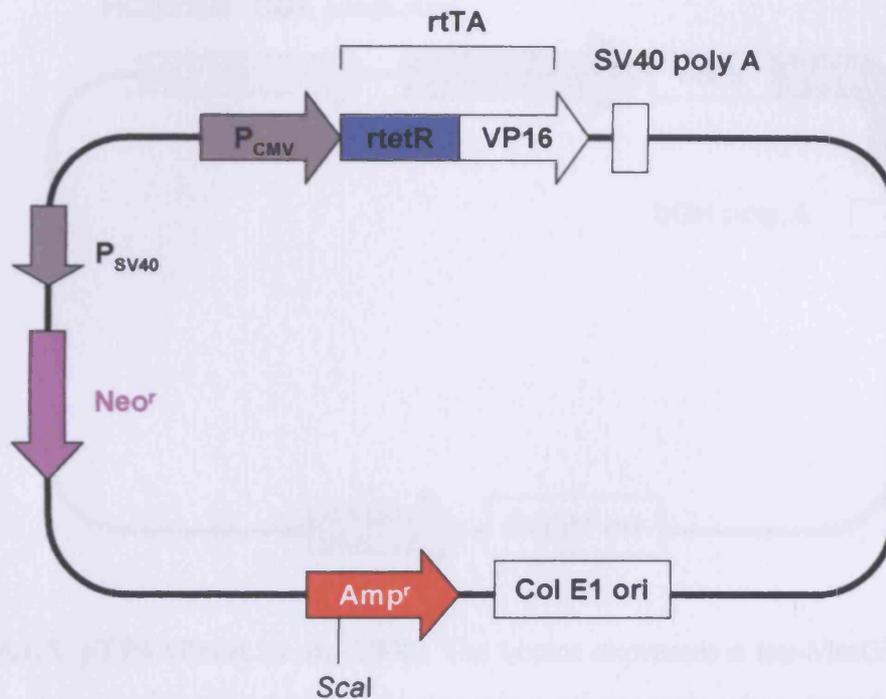


Figure A1.4. pTet-On (BD Biosciences, Oxford, UK) The pTet-On vector contains a strong immediate early promoter of cytomegalovirus (P_{CMV}) from which the reverse tetracycline-responsive transcriptional activator protein, rtTA, is expressed. rtTA is made of a fusion protein between the repressor (rtetR) of the *Tn10* tetracycline resistance operon of *E. coli* and a transcriptional activation domain of the VP16 protein of herpes simplex virus. Cell lines stably transfected with the vector can be selected with neomycin.

A1.5. pTP6

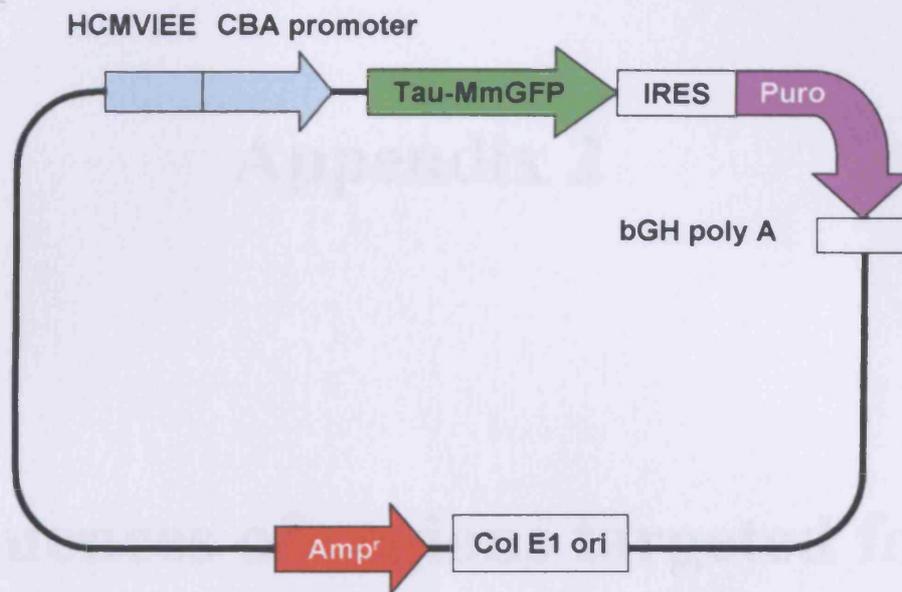


Figure A1.5. pTP6 (Pratt et al., 2000) The vector expresses a tau-MmGFP fusion transgene that can be observed by fluorescence microscopy when transfected into cells. The tau-MmGFP is linked to the puromycin resistance gene by an internal ribosome entry site (IRES) thereby allowing selection of cells stably expressing the vector. The expression of both the tau-MmGFP and the puromycin is driven by a human cytomegalovirus immediate enhancer (HCMVIEE) coupled to a chicken β -actin promoter.

Appendix 2

Sequences of regions targeted for RNAi

Appendix 2: Sequences of regions targeted for RNAi

A2.1. Oct-4 (M34381)

GTGAGCCGTCTTTCCACCAGGCCCGGCTCGGGGTGCCACCTTCCCCATGGCTGG
 ACACCTGGCTTCAGACTTCGCCTCCTCACCCACCAGGTGGGGGTGATGGGTGAG
 AGGGCTGGAGCCGGGCTGGGTGGATTCTCGAACCTGGCTAAGCTTCCAAGGGCCTCC
 AGGTGGGCTGGAATCGGACCAGGCTCAGAGGTATTGGGGATCTCCCCATGTCCGCC
 CGCATAACGAGTTCTGCGGAGGGATGGCATACTGTGGACCTCAGGTTGGACTGGGCCT
 AGTCCCCCAAGTTGGCGTGGAGACTTTGCAGCCTGAGGGCCAGGCAGGAGCACGAGT
 GGAAAGCAACTCAGAGGGAACCTCCTCTGAGCCCTGTGCCGACCGCCCAATGCCGT
 GAAGTTGGAGAAGGTGGAACC**AACTCCCGAGGAGTCCCAGGA**CATGAAAGCCCTGCA
 GAAGGAGCTAGAACAGTTT**GCCAAGCTGCTGAAGCAGAAGAGGATCACCTTGGGGTA**
CACCCAGGCCGACGTGGGGCTCACCTGGGCGTTCTCTTTGGAAAGGTGTTCAGCCA****
GACCACCATCTGTGCTTCGAGGCCTTGCAGCTCAGCCTTAAGAACATGTGTAAGCT
 GCGGCCCTGCTGGAGAAGTGGGTGGAGGAAGCCGACAAC**AATGAGAACC**TT**CAGGA******
GATATGCAAATCGGAGACCCTGGTGCAGGCCCG**AAAGAGAAAGCGAACTAGCATTGA**
 GAACCGTGTGAGGTGGAGTCTGGAGACCATGTTTCTGAAGTGCCGAAGCCCTCCCT
 ACAGCAGATCACTCACATCGCCAATCAGCTTGGGCTAGAGAAGGATGTGGTTCGAGT
 ATGGTTCGTAAACCGCGCCAGAAGGGCAAAGATCAAGTATTGAGTATTC**CCAACG**
 AGAAGAGTATGAGGCTACAGGACACCTTCC**CAGGGGGGGCTGTATCCTTTCCTCTG**
 CCCCAGGTCCC**CACTTTGGCACCCCAGGCTATGGAAGCCCCCACTTACCACACTC**
 TACTCAGTCCCTTTTCTGAGGGCGAGGCCTTCCCTCTGTTCCCGTCACTGCTCTG
 GGCTCTCCCATGCATTCAA**ACTGAGGCACCAGCCCTCCCTGGGGATGCTGTGAGCCA**
 AGGCAAGGGAGGTAGACAAGAGAACCTGGAGCTTTGGGGTTAAATTCTTTTACTGAG
 GAGGGATTAAAGCACAACAGGGGTGGGGGGTGGGATGGGGAAAGAAGCTCAGTGAT
 GCTGTTGATCAGGAGCCTGGCCTGTCTGTCACTCATCATTTTGTCTTAAATAAAGA
 CTGGACACACAGT

Oct-4A siRNA, **Oct-4B siRNA**, **Oct-4 C siRNA**, **psiOct4**

Oct-4 IR_F GGCCACTAGTATG**GAAACAGTTTGCC**

Oct-4 IR_R GGCCTTCGAA**CTTCGGGCACTT**

A2.1 Sequence of Oct-4. The regions highlighted are the regions that were targeted by siRNAs (Chapter 3) or the psiOct4 vector (Chapter 4). The red sequence is the fragment cloned into the pBI-EGFP* vector as an inverted repeat that was amplified using the primers Oct-4 IR_F and Oct-4 IR_R (Chapter 5). These primers were designed so as to add *SpeI* and *BstBI* restriction sites onto the end of the PCR product that could subsequently be used for cloning it.

A2.2. Rex-1 (NM_009556)

ctcctagccgcctagatttccactgtggctctgggtacgagtggcagtttcttcttg
 ggatttcagaaaggaaccaaggaggaaatagagcgcaccgcatcgctgtgggcatt
 aggtaagtcctagaaatggactaagagctgggacacgtggcaaaagaagatagtctt
 cttctctca**atagagtgagtgctgcagtgca**gccagcagctcctgcacacagaagaaa
 gcaggatcgcctcactgtgctgcctccaagtgttgtccccaataaccactgaccaa
 aagcaggtgtttgcggtcagtgccccctggaagtgagtcattaggtgttcaaga
 aggaagcagctaagacaacatgaatgaacaaaaaatgaatgaacaaatgaagaaaac
 ggcaagacaagtggccagaaagggccgggagggaagagccctcgacagactgaccct
 aaagcaagacgaggcaaggccagtcagaaataaccagagtggaagctccccgtgtaac
 atacaccatccgggatgaaagtgagattagccccgagactgaggaagatggcttccc
 tgacggatacctagagtgcatacagaggtgagttttccgaaccattctggaaga
 ggatttccttttaaatccttcgaaagcttgagggaagtggagcaaaaccttctcg
 ccaggttctggaagcaggttcccttctcgagctcttcttggagtacatgacaaaggg
 gacgaagcaagagaagacagaggtcacgcaagagaccctccgctgaggggtggggc
 gagctcattacttgacggcggccctgcagagaagccagagggcggtgtgactgtgg
 tgtcttatcgatgctggagtgtcctcaagccgggtgcaagaagaagctgaggggtaa
 aaccgacctgaggaagcacatgcttgtccacgggccccggcgacgtgtgtgcaga
 gtgtggcaaagccttcacggagagctcgaaactaaagcgacattttctgggtgcacac
 cggagagaagccgtatcagtgcacgttcgaaggctgtgggaagcgtttctccctgga
 ttcaacttgcgacccacatccgcatccacaccggggagagggcgcttctgtgtgccc
 ttttgatggctgcgagaagagctttattcagtcacaataaccagaagattcacatcct
 aaccacgcaaagggcaggaagaaatgctgaaggtggagacagattgtcctcaggct
 gggtagtcttaatgacagaggcactggggatacacgcattatgtacagattgttatt
 tgtagggatagattctatggctaaaataattttgcaactcttcattatacagtttta
 aggcctcttttggtattccatggcatagttccaacaggaaagtgaatggtgaaagca
 taatccttactatatgtgctattgaaggggatggggacttttgcatcggcagaaaa
 tcagttattttaactatgcagttcctaattgcctagcttttaccttagaaatag
 cttcatggtaaatgtgataatcctaagaatggccaactttggcagtagtcaacaaa
 tgaatacatatttctaaattttatgttttgatttttgttttctcattgtaccaaac
 tatattaatcatagttaaatgtgaatagttacaaaaaagttgatccatctgaagatt
 ttagatgtttcaaaaataaaaggaaataaaac

A2.2 Rex-1 The region targeted by the psiRNA_Rex-1 vector (Chapter 4) is highlighted.

A2.3 Laminin B1 (*Lamb1*) (NM_008482)

atggaagggccctctcctctctcccaacatttgcttttctccccgctacctctcc
 agaaaggaagacccgaagaaaagacaggcagcttgctgctgcgtcctccttcccgt
 gccgctcccctcgtctgagaggactggacatggggctgctccaggtgttcgcttt
 ggtgtcctagccctatggggcaccgagtgctgcgctcaggaaccggagttcagctat
 ggctgctcagaaggcagctgctaccctgccactggcgaccttctcatcgggcagcg
 caaaagctctccgtgacttcgacatgtggactgcacaaaccagagccctactgtatt
 gttagccacctgcaggaggacaagaaatgcttcatatgtgactcccagacccttat
 cagagaccctcaaccccgacagccatctcattgagaacgtggtcaccacatttgct
 ccaaaccgcttaagat**ctgggtggcaatcggaaaatgg**gtgggagaacgtgaccatc
 caactggacctggaagcagaattccatttcaactcatctcatcatgacctcaagaca
 ttcgcccagccgcatgctgatcgagcggcttctgactttgggaagacttggggc
 gtgtacagatacttcgctacgactgtgagagctcgttcccaggcatttcaactgga
 cccatgaagaaagtggatgacatcatctgtgactctcgatattctgacattgagccc
 tcgacagaaggagaggtaatatattcgtgctttagatcctgctttcaaaattgaagac
 ccttatagtccaaggatacagaatctattaaaaatcaccaacttgagaatcaagttt
 gtgaaactgcacaccttgggggataaaccttttgactccagaatggaaatccgagag
 aagtactattacgctgtttatgatatgggtggttcgagggaaactgcttctgctatggc
 cacgccagtgaatgcgcccctgtggatggagtcaatgaagaagtggaaggaatggtt
 cacgggactgcatgtgcagacacacaccaaaggcctgaactgtgagctgtgcatg
 gatttctaccacgatttgccgtggagacctgctgaaggccggaacagcaacgcctgc
 aaaaaatgtaactgcaatgaacattccagctcgtgtcactttgacatggcagtcctc
 ctggctactggcaacgtcagcgggggagtggtgtgataactgtcagcacaacaccatg
 gggcgcaactgtgaacagtgcaaaccgcttctacttccagcaccctgagagggacatc
 cgggacccaatctctgtgaacatgtacctgtgacccagctggttctgagaatggc
 gggatctgtgatgggtacactgatttttctgtgggtctcattgctgggtcagtgctcg

A2.3 Part of the sequence of the coding sequence of Laminin B1 (*Lamb1*). The region targeted by the psiRNA_ *Lamb1* vector (Chapter 4) is highlighted. (Regions shown 1-1425 nt, actual coding region from 1- 5504 nt).

A2.4 LaminA/C (LMNA) (X03444)

atggagacccccgtcccagcggcgcgccacccgcagcggggcgcaggccagctccact
 ccgctgtcgcccacccgcatcaccgggctgcaggagaaggaggacctgcaggagctc
 aatgatcgcttggcggtctacatcgacctgtgctgctcgctggaaacggagaacgca
 gggctgcgcttcgcatcaccgagctctgaagaggtggtcagccgcgaggtgtccggc
 atcaaggccgctacgaggccgagctcggggatgcccgaagacccttgactcagta
 gccaaggagcgcgcccgcctgcagctggagctgagcaaagtgcgtgaggagttaag
 gagctgaaagcgcgcaataccaagaaggaggggtgacctgatagctgctcaggctcgg
 ctgaaggacctggaggctctgctgaactccaaggaggccgcactgagcactgctctc
 agtgagaagcgcacgctggagggcgagctgcatgatctgcggggccaggtggccaag
 cttgaggcagccctaggtgaggccaagaagcaacttcaggatgagatgctgcgggcgg
 gtggatgctgagaacaggctgcagaccatgaaggaggaactggacttcagaagaac
 atctacagtgaggagctgctgagaccaagcgcctcatgagacccgactggtggag
 attgacaatgggaagcagcgtgagtttgagagccggctggcggatgctgctgcaggaa
 ctgcggggccagcatgaggaccaggtggagcagataagaaggagctggagaagact
 tattctgccaaagctggacaatgccaggcagctctgctgagaggaacagcaacctggtg
 ggggctgcccacgaggagctgcagcagctcgcgcatccgcatcgacagcctctctgcc
 cagctcagccagctccagaagcagctggcagccaaggaggcgaagcttcgagacctg
 gaggactcactggcccgtgagcgggacaccagccggcggctgctggcggaaaaggag
 cgggagatggccgagatgcgggcaaggatgcagcagcagctggacgagtaccaggag
 ctctggacatcaagctggccctggacatggagatccacgcctaccgcaagctcttg
 gaggcgaggaggagaggctacgcctgtccccagccctacctcgacgcgagccgt
 ggccgtgcttctctcactcatcccagacacaggggtgggggcagcgtcaccaaaaag
 cgaaactggagtccactgagagccgcagcagcttctcacagcacgcacgactagc
 gggcgcgtggccgtggaggaggtggatgaggagggcaagtttgtccggctgcgcaac
 aagtccaatgaggaccagctccatgggcaattggcagatcaagcgccagaatggagat
 gatcccttgcctgacttaccgggtcccaccaaagtccaccctgaaggctgggcaggtg
 gtgacgatctgggctgcaggagctggggccaccacagccccctaccgacctggtg
 tggaaaggcacagaacacctggggctgcgggaacagcctgctgctacggctctcatcaac
 tccactggggaagaagtggccatgcgcaagctggtgctcagtgactgtggttgag
 gacgacgaggatgaggatggagatgacctgctccatcaccaccacggctcccactgc
 agcagctcgggggaccccgctgagtaaacctgcgcctcgcgaccgtgctgtgcgg
 gacctgcgggcagcctgccgacaaggcatctgccagcggctcaggagcccaggtggg
 cggaccatctcctctggctcttctgcctccagtgtcacggctactcgcagctaccg
 cagtgtggggggcagtggggggtggcagcttcggggacaatctggtcaccgcctccta
 cctcctgggcaactccagccccgaaccagagccccagaactgcagcatcatgta
 atctgggacctgccaggcaggggtgggggtggaggcttctgctcctcctcacctc
 atgcccacccccctgccctgcagctcatgggagggggcttgaagccaaagaaaaataa

LMNA IR_F GGCCACTAGTATGAGATGCTGCGGC

LMNA IR_R GGCCTTCGAACTCACGCTGCTTCCC

A2.4 Sequence of the human Lamin A/C coding region (LMNA). The region highlighted is the fragment cloned into the pBI-EGFP* vector as an inverted repeat that was amplified using the primers LMNA IR_F and LMNA IR_R (Chapter 5). These primers were designed so as to add *SpeI* and *BstBI* restriction sites onto the end of the PCR product that could subsequently be used for cloning it.

Appendix 3

Cell Area Measurements

Appendix 3: Cell Area Measurements

Area of Experiment cells (μm^2)				Area of Control cells (μm^2)			
173	941	1562	2558	161	389	754	1711
199	960	1594	2633	175	389	766	1752
278	972	1610	2657	182	391	794	1766
326	989	1627	2873	194	406	826	1783
372	991	1642	2899	211	430	852	1798
382	1008	1726	3106	214	439	886	1850
396	1049	1752	3221	235	470	895	1894
442	1073	1769	3245	238	485	905	2023
478	1092	1776	3262	240	490	922	2095
538	1157	1855	3329	242	583	926	2179
540	1224	1874	3389	245	600	1027	2249
571	1248	1913	3466	262	605	1032	2338
617	1253	2042	3511	271	614	1032	2678
631	1310	2052	3571	276	617	1056	2798
634	1358	2057	3732	290	638	1068	2842
636	1361	2100	4296	295	667	1123	2957
646	1375	2170	4399	302	670	1169	3398
660	1380	2174	4447	305	689	1205	3670
703	1390	2189	4447	307	694	1250	3842
715	1416	2215	4769	312	715	1387	5189
720	1426	2266	4834	314	715	1394	5534
797	1445	2393	5148	322	718	1462	5558
833	1450	2429	5249	346	739	1507	7416
898	1481	2486	5422	362	742	1627	7589
898	1481	2534	13174	370	754	1699	9816

Table A3.1 Cell measurements for Experiment 1 (Chapter 4)

Area of Experiment cells (μm^2)				Area of Control cells (μm^2)			
286	1268	2659	4295	123	245	286	409
368	1309	2699	4335	123	245	286	409
368	1350	2699	4908	123	245	327	450
409	1350	2822	4908	164	245	327	450
450	1391	2863	5235	164	245	327	450
450	1513	2863	5440	164	245	327	450
532	1636	2904	5522	164	245	327	491
573	1677	2986	5726	164	245	327	491
573	1718	3108	5726	164	245	327	532
573	1759	3149	5767	205	245	327	532
614	1800	3231	6299	205	245	327	654
695	1881	3313	6544	205	245	327	695
736	1922	3354	6585	205	245	368	736
777	1922	3354	6626	205	286	368	1023
818	1963	3436	7198	205	286	368	1186
859	2086	3558	8139	205	286	368	1227
900	2127	3640	8712	205	286	368	1432
982	2168	3681	9489	205	286	368	1677
982	2209	3763	9939	205	286	409	1718
982	2209	3886	10961	205	286	409	3436
1145	2495	3926	11288	245	286	409	4254
1145	2536	4049	11370	245	286	409	4540
1186	2536	4049	12229	245	286	409	4622
1186	2618	4049	15256	245	286	409	5072
1227	2659	4172	22086	245	286	409	6340

Table A3.2. Cell measurements for Experiment 2 (Chapter 4)

Area of Experiment cells (μm^2)				Area of Control cells (μm^2)			
286	1104	1800	3149	164	286	450	1391
368	1145	1800	3272	164	286	450	1432
368	1145	1963	3477	164	327	450	1472
409	1268	2004	3517	164	327	450	1513
450	1309	2045	3640	164	327	491	1595
450	1309	2045	3763	205	327	532	1636
450	1309	2086	3886	205	327	573	1636
450	1309	2127	4008	205	327	573	1677
450	1350	2168	4008	205	327	654	1677
450	1350	2209	4254	205	327	654	1759
532	1391	2209	4458	205	327	736	2045
532	1432	2250	4663	205	327	859	2209
573	1472	2290	5194	205	327	982	2290
614	1472	2331	5194	245	327	982	2331
736	1554	2372	5235	245	368	1023	2699
736	1554	2413	5481	245	368	1063	3722
736	1636	2454	6135	245	368	1063	3804
859	1636	2495	6135	245	368	1145	4008
900	1636	2618	6258	245	368	1145	4008
900	1636	2659	7076	245	368	1145	4335
900	1636	2659	7158	286	368	1186	4622
941	1677	2740	7403	286	368	1227	7935
941	1759	2863	7403	286	409	1268	8712
982	1759	2945	8221	286	409	1309	11616
1023	1759	3027	13906	286	409	1309	11657

Table A3.3 Cell measurements for Experiment 3 (Chapter 4)

Area of Experiment cells (μm^2)				Area of Control cells (μm^2)			
164	491	1063	1963	123	245	327	614
205	491	1104	2127	123	245	327	654
205	532	1104	2290	164	245	327	654
245	532	1145	2413	164	245	327	654
245	532	1186	2413	164	245	327	654
286	573	1186	2495	164	245	327	654
286	573	1268	2659	164	245	368	736
286	573	1309	2659	164	245	368	736
327	573	1309	2659	164	245	368	777
327	614	1513	2740	205	245	409	818
327	614	1513	2863	205	245	409	982
368	654	1554	3231	205	245	450	1104
368	654	1554	3395	205	286	450	1104
409	654	1636	3477	205	286	450	1227
409	695	1636	3640	205	286	450	1309
409	736	1718	3926	205	286	450	1391
409	736	1718	4295	205	286	491	1432
450	777	1759	4376	205	286	491	1800
450	777	1800	5440	205	286	491	1841
450	777	1800	6626	205	286	532	2127
450	818	1841	8262	205	286	532	3354
450	900	1881	8425	245	286	614	3926
450	941	1881	8712	245	286	614	4213
450	941	1881	9448	245	327	614	6012
450	1063	1922	9980	245	327	614	15215

Table A3.4 Cell measurements for 1:10 Transfection (Chapter 4)

Area of Experiment cells (μm^2)				Area of Control cells (μm^2)			
123	614	1922	3845	123	205	327	654
123	614	1963	3886	164	205	327	654
123	654	2004	3926	164	205	327	654
164	736	2250	4049	164	205	327	695
205	736	2290	4090	164	245	327	695
205	777	2331	4131	164	245	327	777
245	859	2413	4213	164	245	327	777
245	859	2495	4295	164	245	327	818
245	900	2536	4295	164	245	327	859
245	982	2577	4499	164	245	327	859
245	982	2577	4704	164	245	327	900
245	1023	2822	4744	164	245	327	1063
245	1023	2822	4785	205	245	368	1268
286	1063	2986	4867	205	245	368	1268
286	1145	3027	4867	205	245	368	1432
286	1554	3027	5153	205	245	368	1595
327	1595	3068	5317	205	286	368	1800
409	1595	3149	5358	205	286	368	1841
409	1636	3149	5522	205	286	368	2168
450	1677	3272	6176	205	286	368	3068
450	1718	3395	6340	205	286	409	4049
491	1800	3436	7198	205	286	409	4049
573	1881	3517	10879	205	286	532	5031
573	1922	3558	11697	205	286	573	5481
614	1922	3640	11902	205	286	573	6544

Table A3.5 Cell measurements for 1:3 Transfection (Chapter 4).

Area of Experiment cells (μm^2)				Area of Control cells (μm^2)			
205	900	1595	3395	164	286	409	982
205	941	1595	3436	164	286	450	1023
245	941	1636	3517	164	286	450	1104
327	1023	1636	3517	205	286	450	1595
368	1023	1677	3558	205	286	450	1677
368	1023	1759	3558	205	286	491	1677
368	1023	1759	3599	205	286	491	1677
409	1063	1759	3599	205	286	532	1718
409	1063	1800	3681	205	286	532	1759
409	1063	1800	4008	205	327	532	1841
450	1063	2127	4090	205	327	532	2127
450	1104	2209	4744	205	327	532	2331
491	1145	2372	4867	205	327	573	2863
532	1145	2413	4867	205	327	573	2986
614	1186	2536	5031	205	327	573	3068
614	1186	2577	5153	205	368	614	3763
654	1227	2618	5358	245	368	654	4090
695	1268	2659	5890	245	368	654	4335
695	1309	2740	6380	245	368	654	4335
695	1309	2822	6544	245	368	736	4458
695	1350	2863	6912	245	368	736	4785
736	1554	2986	6953	245	368	777	4867
736	1554	3190	7198	245	409	777	6217
777	1554	3231	7280	245	409	818	7035
777	1554	3354	10511	245	409	941	15378

Table A3.6 Cell measurements for 1:1 Transfection (Chapter 4)

