The Role of the Anterior Endoderm in Cardiac Specification

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To Neesha, Alexander and James

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ABSTRACT

The heart is the first functional organ of embryogenesis in many vertebrates, however little is known about the early specification events of cardiogenesis. Evidence in the chick and amphibian suggests a requirement for the anterior endoderm in cardiac induction to direct mesoderm toward a cardiac fate. Furthermore, the signals responsible for specification and their mode of action are unknown. Several signalling pathways, including FGF, Nodal, BMP and Wnt have been implicated. However, as these pathways have other roles in early embryogenesis a specific role in cardiac induction has been difficult to define. We have devised a model testing the cardiac-inducing activity of the anterior endoderm addressing its ability to re-specify pluripotent embryonic ectoderm upon conjugation.

We have shown that the anterior endoderm is sufficient to induce robust expression of cardiac markers and formation of contractile tissue in the responder. Characterisation of the model showed the anterior endoderm produces a specific signal; skeletal muscle is not induced, distinguishing it from general mesoderm induction. The cardiac-inducing capacity of the anterior endoderm was not uniform as it was restricted to the most anterior regions of the anterior endoderm, correlating with expression of *Hex*. The cardiac-inducing signal requires two hours of interaction with the responding tissue during gastrulation to produce an effect. Further involvement of the anterior endoderm beyond specification of cardiac precursors was not required.

The model provided the basis to investigate the early signalling events of specification. Whereas BMP signalling was not necessary for cardiac induction by the endoderm, an essential requirement for FGF and Nodal pathways was shown. Timed inhibition revealed both were required during the first hour of conjugation, while sustained ERK activation was needed for at least four hours. In addition it was shown that elevated Wnt/β-catenin signalling during specification had no effect, while sustained activation antagonised cardiogenesis. Further analysis revealed Wnt/β-catenin has no direct role in specification, but suppression or low activity was required prior to the onset of cardiac differentiation. Therefore, this work established a simple and experimentally amenable assay for elucidating the mechanisms of cardiac specification.

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LIST OF ABBREVIATIONS

AC	Animal Cap
AC/AE	Animal Cap/Anterior Endoderm Conjugate
AC/PE	Animal Cap/Posterior Endoderm Conjugate
AE	Anterior Endoderm
ALK	Activin receptor like Kinase
ANF	Atrial Natriuretic Factor
A-P	Anterior-Posterior
AVE	Anterior Visceral Endoderm
bFGF	basic Fibroblast Growth Factor
bHLH	basic Helix Loop Helix
BMP	Bone Morphogenetic Protein
BMPR	Bone Morphogenetic Protein Receptor
BSA	Bovine Serum Albumin
caALK4	constituively active Activin receptor like Kinase
CAc	Cardiac Actin
CaMKII	Calmodulin dependent protein Kinase II
Cerb	Cerberus
CerbMO	Cerberus Morpholino
CerS	Cerberus Short
CMFM	Calcium-Magnesium Free Medium
CNS	Central Nervous System
coSmad	common Smad
CSKA	Cytoskeletal actin
CTnI	Cardiac Troponin
DAN	differential screening-selected gene aberrative in neuroblastoma
Dex	Dexamethasone
dH_20	Distilled Water
Dkk-1	Dickopf-1
DMSO	Dimethyl Sulphoxide
DMZ	Dorsal Marginal Zone
dNTP	deoxyribonucleotide triphopshate
dpERK	diphosphorylated ERK
Dpp	decapentaplegic
Dsh	Dishevelled
DTT	Dithiothreitol
D-V	Dorsal-Ventral
ECM	Extracellular Matrix
Edd	Endodermin
EnR	Engrailed Repressor
Eomes	Eomesodermin
ERK	Extracellular signal-Regulated Kinase
ES	Embryonic Stem Cell
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
Frz	Frizzled
FXR	Farnesoid-X-Receptor

GR	Glucocorticoid Receptor
Gsc	Goosecoid
GSK3β	Glycogen Synthase Kinase 3β
h	Hours
HA	Haemaglutinin
HcG	Human chorionic Gonadotrophin
HexMO	Hex Morpholino
HSS	Heat inactivated Sheep Serum
IFABP	Intestinal Fatty Acid Binding Protein
Ig	Immunoglobulin
iPS	induced Pluripotent Stem Cell
Irx4	Iroquois
ISmad	Inhibitory Smad
JNK	c-Jun Kinase
LCMR	Low Calcium-Magnesium Ringer's Medium
LEF	Lymphoid Enhancer binding Factor
LFABP	Liver Fatty Acid Binding Protein
LiCl	Lithium Chloride
LRP	lipoprotein receptor-related protein
LURP-1	Ly-6/uPAR-related protein
M	Molar
MAB-T	Maleic Acid Buffer-Triton
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MBT	Mid-Blastula Transition
MEMFA	MOPS-EDTA-Magnesium Formaldehyde
mES	murine Embryonic Stem cell
mg	milligram
MHCα	Mysoin Heavy Chain α isoform
min	Minute
Mixer	Mix-like endodermal regulator
ml	millilitre
MLC	Myosin Light Chain
mM	millimolar
MO	Morpholino
MSR	Mesenchyme associated Serpetine Receptor
Myf5	Myogenic Factor 5
MyoD	Myogenic Determination
MZ	Marginal Zone
NAM	Normal Amphibian Media
Ntub	N-Tubulin
ng	nanogram
ODC	Ornithine Decarboxylase
Oep	One-eyed Pinhead
pAC	peeled Animal Cap
PBT	Phosphate Buffered Triton
PCP	Planar Cell Polarity
Pdx1	Pancreatic and duodenal homeobox 1
pg	picogram
PI_3	Phospho Inositol Triphosphate

PKC	Protein Kinase C
PLC	Phospholipase C
PLP	Posterior Lateral Plate
PPS	Posterior Primitive Streak
PVDF	Polyvinylidene Fluoride
PVP	Polyvinylpyrrolidone
rpm	revolutions per minute
RSmad	Regulatory Smad
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
RXR	Retinoid-X-Receptor
Scl	Stem-cell leukaemia
SDS	Sodium Dodecyl Suplhate
sFRP	secreted Frizzled Related Protein
SmActin	Smooth Muscle Actin
TBE	Tris Buffered EDTA
tBr	truncated BMP Receptor
TBS	Tris Buffered Saline
TCF	T-cell transcription Factor
TGFβ	Tumour Growth Factor
TTw	Tris Buffered Saline-Tween
UV	Ultraviolet
V	Voltage
VMZ	Ventral Marginal Zone
WE	Whole Embryo
WMISH	Whole Mount in *situ* Hybridisation
Wnt	Wingless-type MMTV integration site
Xbra	Xenopus Brachyury
XCR	Xenopus-Cripto Related
Xnr	Xenopus Nodal Related
XPDIp	Xenopus Pancreatic Disulphide Isomerase protein
Xpo	Xenopus Posterior
XVent2	Xenopus Ventral 2
ΔActRIIB	dominant-negative Activin Receptor IIB
ΔFGFR1	dominant-negative Fibroblast Growth Receptor
ΔTCF3	dominant-negative T-cell transcription Factor 3
μg	microgram
μl	microlitre
μM	micromolar

CHAPTER 1 - INTRODUCTION

1.0 INTRODUCTION

Development of the zygote results in the differentiation of hundreds of diverse cell types that make up the body. How this diversity arises from a single cell and what mechanisms are involved in differentiation is one of the key questions of developmental biology (Gilbert, 2006). Following specification, a process by which naïve cells are induced to enter a developmental pathway resulting in the appearance of a definitive cell type, differentiated tissues that are structurally and functionally distinct from one another are not simply distributed but are organised in an orderly manner by a process of morphogenesis. A specified cell may not ultimately form the tissue that it is has been directed toward until it is determined, by which point the specified cells express cell-specific proteins that confirm its particular identity (Slack, 1991b). Specification results in a cell expressing a particular subset of genes that controls when and where proteins are synthesised. The vast numbers of genes establish complex intracellular networks between interacting proteins and genes, and between proteins and proteins to confer properties of a particular cell fate. One of the major mechanisms by which cell fate decisions are made to generate diversity during development is via cell-cell signalling, or ***induction*** (Wolpert *et al.*, 2007). The process of embryonic induction is the interaction between one tissue and another, in which one cell type acts as an inducer that directs the responder to undergo a change in direction of cell fate, resulting in the transcriptional activation of new genes (Gurdon, 1987).

1.1 Embryonic Patterning in Xenopus laevis

1.1.1 Xenopus laevis as a Developmental Model

The South African clawed frog *Xenopus laevis* is an important research model of embryonic development. It became popular after it was realised that ovulation in this species can be induced by injection of Human Chorionic Gonadotrophin (HcG); this permitted a steady supply of embryos in contrast to seasonal availability of embryos of other amphibian species that were used in research. These embryos undergo well-documented stage development as shown in figure 1.1.

Xenopus embryos can be obtained in large numbers (1-2,000/clutch) and develop rapidly in simple salt media. The embryos are very robust and are well suited for experimental embryology approaches. Examples of the contribution of *Xenopus* as a developmental model include formation of embryonic fate maps and elucidation of major families of inductive signalling molecules conserved across all vertebrates (Beck and Slack, 2001). The large embryos (~1mm in diameter) are easily manipulated, with dissection and grafting experiments very applicable techniques (Gurdon *et al.*, 1984; Kuroda *et al.*, 2004; Nieuwkoop, 1969). Analysis of gene function in *Xenopus* is easily achieved through overexpression of mRNA and gene knockdown using antisense morpholino (MO) techniques. MOs are designed to specifically target RNA, causing steric hindrance and prevent translation of the protein. In addition, they can be used to knockout certain regions by their ability to block splicing events of pre-mRNA (Heasman, 2002; Heasman *et al.*, 2000). mRNA encoding a gene of interest is easily injected into the large blastomeres of the early embryo and its ability to be translated has led to positive functional analysis of key molecules in development (Cho *et al.*, 1991; Glinka *et al.*, 1998; Sasai *et al.*, 1994; Smith *et al.*, 1993). Over recent years, its use as a developmental model has increased due to the *Xenopus* genome initiative, providing a vastly improved annotated database, 'Xenbase'. Due to these attributes, *Xenopus* has been adopted as the model for this study with potential benefits of this model described. Thus, subsequent passages describe the features of *Xenopus* development and how they relate to processes in other vertebrates.

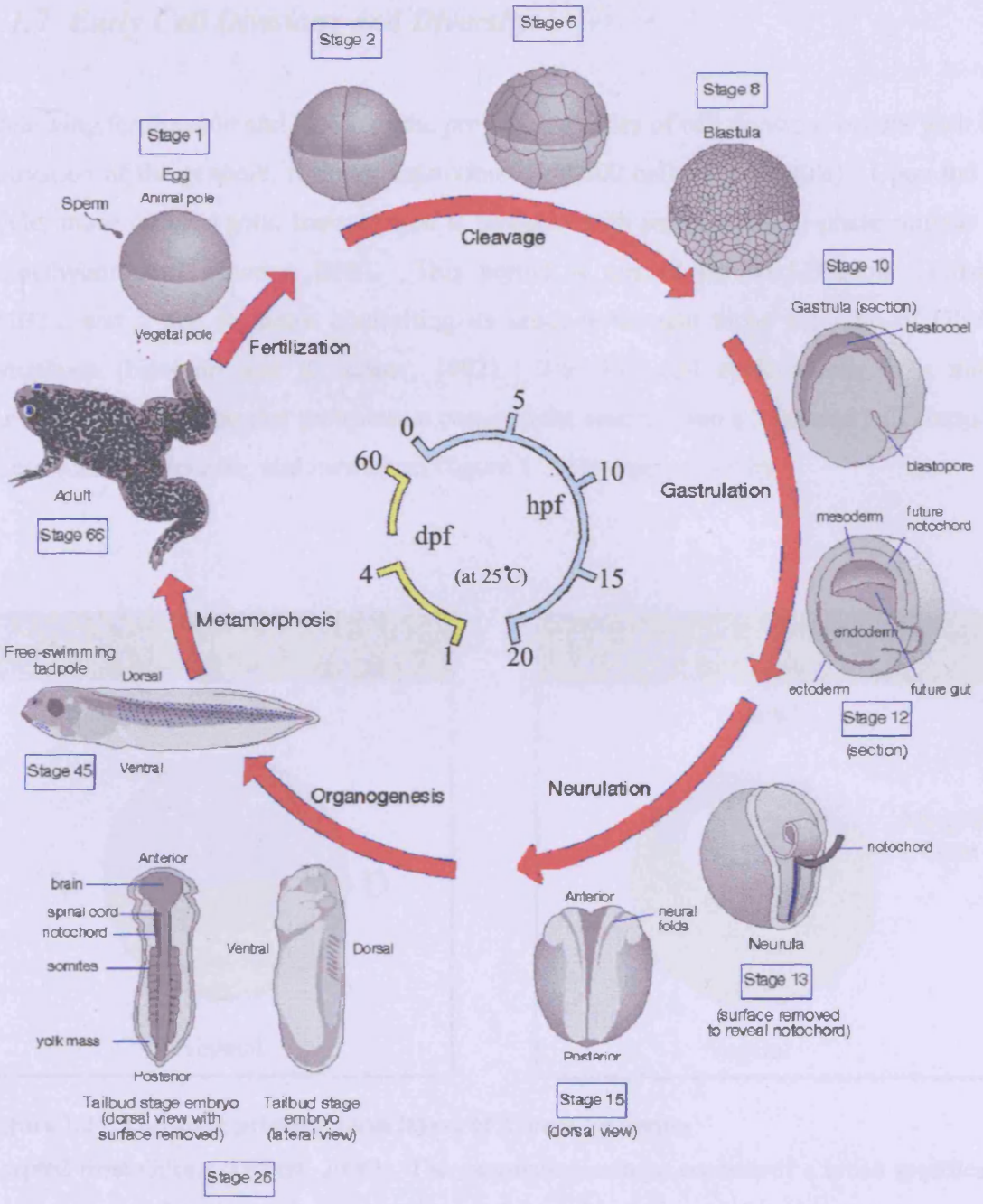


Figure 1.1 – Xenopus Stage Development

Taken from (Wolpert *et al.*, 2007), *Xenopus* have a well documented stage of development. Following fertilisation, the embryo undergoes many cleavages forming a blastula (stage 1-10). Upon activation of zygotic transcription, the embryo undergoes complex involuting events (gastrulation, stage 10.5). This is followed by neural plate and neural fold formation, leading on to organogenesis and tadpole formation. After approximately four days the tadpole begins to metamorphose, resulting in an adult frog after 3 to 6 months.

1.1.2 Early Cell Divisions and Diversity

Following fertilisation and fusion of the pronuclei a series of cell divisions occurs with little activation of the genome, forming approximately 4,000 cells (the blastula). Upon the 12th cycle, more rapid zygotic transcription is initiated with inclusion of G-phase mitosis and demethylation of genomic DNA. This period is termed the Mid-Blastula Transition (MBT), and a key regulator controlling its onset is thought to be the ratio of DNA to cytoplasm (Newport and Kirschner, 1982). The 15th cell cycle results in a mitotic quiescence and subsequent gastrulation converts the embryo into a 3 layered ball composed of endoderm, ectoderm, and mesoderm (figure 1.2; Heasman, 2006b).

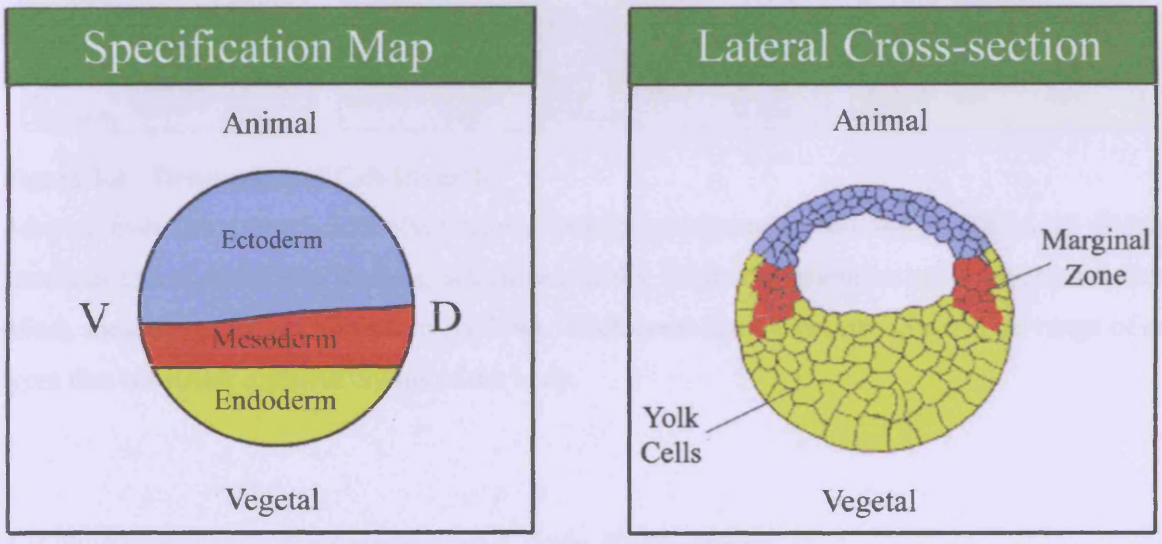


Figure 1.2 – The three primary germ layers of *Xenopus* gastrula

Adapted from Gilbert (Gilbert, 2006). The gastrulating embryo consists of a broad specification map composed of the three primary germ layers; the ectoderm, the mesoderm, the endoderm.

It is upon commencement of germ layer formation that early cell diversity in the embryo becomes apparent. One of the earliest methods of diversification of cell type arises from the fact that the particular fate of a cell may be determined by the layer in which it arises, be it the animal region of the embryo (ectoderm), vegetal region (endoderm) or the marginal region (mesoderm) (figure 1.3; Gilbert, 2006). Each germ layer however, does not merely give rise to one type of cell. Instead, complex patterning and induction results

in formation of many different cell types from a common progenitor, inevitably forming multiple tissues that express distinct genes unique to that lineage. All cells (except those of the germline) contain the same genes, so how exactly a particular tissue expresses a unique subset of genes is the main focus of early embryology (Gilbert, 2006).

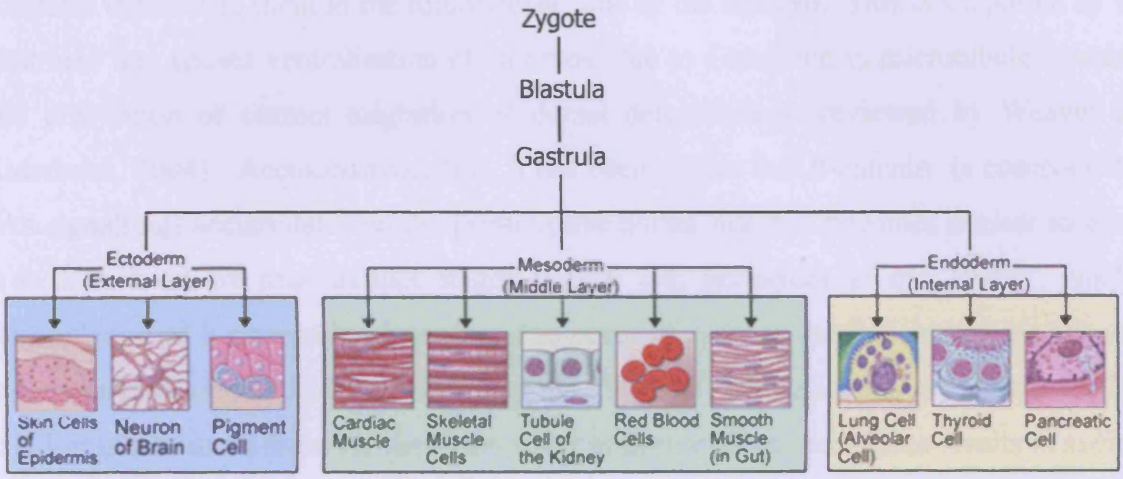


Figure 1.3 - Generation of Cell Diversity

Adapted from (http://www.ncbi.nlm.nih.gov/About/primer/genetics_cell.html.). Early cell division generates the multicellular blastula, which eventually forms the primitive germ layers; ectoderm (blue), mesoderm (green), endoderm (yellow). Each germ layer gives rise to a diverse range of cell types that constitute different organs of the body.

1.1.3 Embryonic Patterning and Axis Formation

Patterning of the embryo results in formation of a well-ordered body plan, resulting from careful spatial and temporal organisation of cellular activity. In vertebrate development a conserved body plan exists with two distinct axes; the anterior-posterior (A-P) axis from head to tail, and the Dorsal-Ventral (D-V) axis from back to belly. Establishment of the D-V axis is the first occurrence of patterning in the early embryo, and is the result of localisation of dorsal-determinants on the presumptive dorsal side of the embryo (reviewed by De Robertis and Kuroda, 2004). The exact identification of these dorsal determinants is not known but is thought to contain components of Wingless-type MMTV integration site (Wnt) signalling (reviewed in section 1.1.5). Sperm entry upon fertilisation results in a process of cortical rotation, where the cortex rotates relative to the yolky core cytoplasm.

As a result, sub-cellular structures are propelled toward the future dorsal side approximately 180° from the sperm entry point (figure 1.4). This alignment of organelles is thought to establish a microtubule organisation that transports vesicles of dorsal determinants to one side of the embryo (Rowning *et al.*, 1997), with the sperm centriole at the point of entry acting as a microtubule organising centre. It is thought that movement of determinants is then achieved via kinesin dependent motors, with the microtubule assembly essential to correctly translocate them to the future dorsal side of the embryo. This is supported by UV treatment that causes ventralisation of embryos, due to disruption in microtubule assembly and prevention of correct migration of dorsal determinants (reviewed by Weaver and Kimelman, 2004). Accompanying this, it has been shown that β-catenin (a component of Wnt signalling) accumulates on the presumptive dorsal side and becomes nuclear localised at a peak level by mid-blastula stages (figure 1.4; Schneider *et al.*, 1996). Nuclear accumulation of β-catenin has been found to occur as early as the first embryonic cleavage and accumulates toward MBT (Larabell *et al.*, 1997). This localisation is essential for D-V axis formation, as its maternal depletion using antisense oligonucleotides results in radially symmetrical embryos lacking all anterior, posterior, and ventral structures (Heasman *et al.*, 1994). Antisense oligonucleotides are designed to specifically target maternal mRNA, which activates endogenous RNase H that cleaves the RNA-DNA duplex. The result is the loss of the maternal mRNA transcripts but importantly does not effect zygotic transcription (Dash *et al.*, 1987). Furthermore, overexpression of a mutated version of *TCF3,* whose normal function is to form part of a transcriptional enhancer complex bound by β-catenin to drive gene expression, blocks endogenous axis specification (Molenaar *et al.*, 1996). This is consistent with the evidence which suggests that dorsal determinants include mediators of Wnt signalling, such as *Dishevelled* (*Dsh*) and *Glycogen Synthase Kinase 3β* (*GSK3β*)-*binding protein* (*GBP*), that permit accumulation of β-catenin (Weaver and Kimelman, 2004).

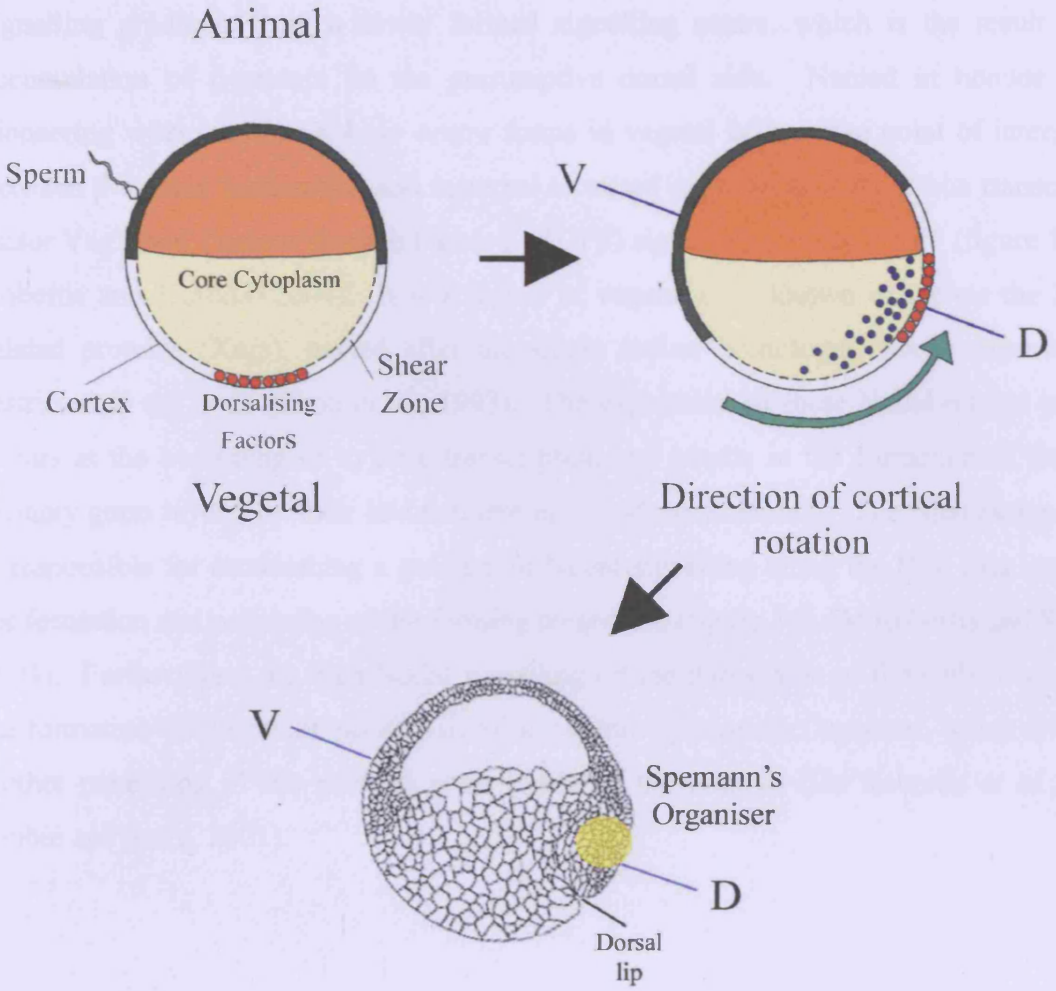


Figure 1.4 – Cortical rotation and localisation of β-catenin in D-V axis formation

Adapted from Wolpert (2007), sperm entry redistributes dorsalising factors essential for D-V axis formation. After fertilisation, the cortex rotates and a microtubule network is established that relocates dorsalising determinants (including *Wnt11* and Dishevelled; red dots) opposite the point of sperm entry. Nuclear accumulation of the intracellular Wnt factor β-catenin ensues (blue dots), and is found to be essential to establish a group of cells referred to as the *Nieuwkoop* centre. This signalling centre is required to initiate germ layer formation and induction of the gastrula signalling centre, Spemann's Organiser (yellow region).

Formation of the D-V axis is accompanied by establishment of characteristic D-V signalling gradients from a newly formed signalling centre, which is the result of the accumulation of β-catenin on the presumptive dorsal side. Named in honour of his pioneering work, the *Nieuwkoop centre* forms in vegetal cells at the point of intersection between β-catenin localisation and maternal localised expression of the T-box transcription factor VegT and Tumour Growth Factor β (TGFβ) signalling molecule Vg1 (figure 1.5; De Robertis and Kuroda, 2004). It is a region of vegetal cells known to secrete the Nodal-related proteins (Xnrs), named after the single mouse homologue whose expression is restricted to the node (Zhou *et al.*, 1993). The expression of these Nodal-related proteins occurs at the beginning of zygotic transcription and results in the formation of the three primary germ layers, by their involvement in mesoderm induction. The Nieuwkoop centre is responsible for establishing a gradient of Nodal signalling along the D-V axis important for formation and patterning of the forming mesoderm (figure 1.5; De Robertis and Kuroda, 2004). Furthermore, the high Nodal signalling on the dorsal side of the embryo results in the formation of the potent gastrula signalling centre Spemann's Organiser, which is vital in further patterning of the primary germ layers of the embryo (De Robertis *et al.*, 2000; Joubin and Stern, 2001).

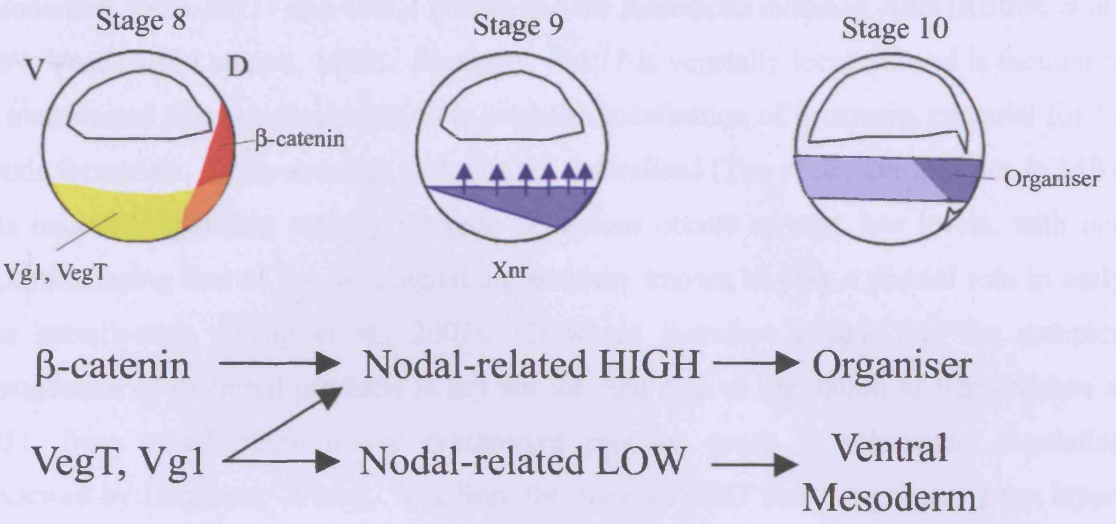


Figure 1.5 – Three primary germ layers of the *Xenopus* embryo

Adapted from Agius *et al.* (2000). The *Nieuwkoop* centre forms at the point of intersection between dorsally localised β-catenin and maternally localised VegT and Vg1. The centre then induces expression of the Nodal-related ligands, generating a gradient from Dorsal to Ventral. This is thought to pattern the mesoderm, with high levels of Nodal inducing the Organiser and subsequently dorsal mesoderm.

Formation of a patterned embryo begins long before the establishment of the three primary germ layers, and as already discussed maternal factors contribute toward early patterning. The unfertilised oocyte contains many maternally expressed mRNAs and proteins which are predicted to exhibit some regional specific localisation given the subsequent expression patterns of genes that depend upon on these factors. Evidence for the importance of maternally inherited transcripts has been achieved by studying the consequence of depleting these transcripts on development (Wylie and Heasman, 1997). It has been shown that a pre-patterning of the embryo exists with localisation of different transcriptional activators/repressors and signalling molecules in such a way that they are specifically inherited by different regions of the embryo (Heasman, 2006b). For example, VegT and Wnt11 are known to be important for germ layer formation and axis specification (reviewed by White and Heasman, 2008). VegT is a T-box transcription factor localised to the vegetal cells of the embryo capable of inducing endoderm and mesoderm in animal caps (Zhang and King, 1996). \when depleted, endoderm and mesoderm formation is severely affected

(Clements et al., 1999), which is thought to be the result of its subsequent regulation of the endodermal genes *Sox17* and *GATA* factors and the mesoderm inducing Xnrs (Kofron et al., 1999; Yasuo and Lemaire, 1999). Similarly, *Wnt11* is vegetally localised and is thought to be translocated upon cortical rotation to establish localisation of β-catenin, essential for D-V axis formation. In its absence, embryos are ventralised (Tao *et al.*, 2005). Prior to MBT it is believed signalling activity between cells does occurs at very low levels, with one exception being that of the Wnt signalling pathway known to play a pivotal role in early axis specification (Yang et al., 2002). It would therefore appear that the complex arrangement of maternal products is key for the first step of regulation of transcription at MBT, from which these newly synthesised proteins result in subsequent regulation (reviewed by Heasman, 2006a). It is from the onset of MBT that the primary germ layers begin to form (figure 1.2).

1.1.4 Mesendoderm Specification and Germ Layer Formation

By the end of gastrulation the embryo is composed of three distinct layers. Each of these germ layers possess a broad specific competence that permits cells within them to adopt certain fates depending upon the germ layer in which they reside; cells of the ectoderm comprising the epidermis and Central Nervous System (CNS), cells of the endoderm forming digestive tract and its derivatives such as liver, and cells of mesoderm forming internal organs, skeletal tissue, muscle, connective tissue and blood (Gilbert, 2006). At blastula stages, explants of vegetal tissue cultured in isolation develop into endodermal tissues, and explants near the animal pole form epidermal cells. In *Xenopus,* endoderm and ectoderm are also specified by maternal factors and mesoderm formation commences upon the beginning of zygotic transcription. The onset of MBT and initiation of zygotic transcription induces mesendoderm formation, and partly depends upon maternal information along the Animal-Vegetal axis established during oogenesis (Yasuo and Lemaire, 2001).

1.1.4.1 Mesoderm induction is initiated by signals from the vegetal region

The understanding of how mesodermal tissue is formed stems from work by Nieuwkoop and colleagues. It was shown that upon conjugating blastula stage vegetal pole to animal

cap (AC) cells, a variety of mesodermal tissues were induced including notochord, muscle, blood, and mesenchyme. Normally, neither AC nor vegetal pole expresses such tissue. Therefore, the vegetal pole was deemed to be inducing mesoderm in the AC and directing it away from its epidermal fate (Nieuwkoop, 1969). The timing of specification events was initially determined by conjugating vegetal poles of different age with blastula stage AC. It was found that mesoderm inducing capacity of the vegetal region is lost by mid-gastrula stages (stage 10.5 to 11, approximately 10 hours after fertilisation; Gurdon *et al.*, 1985; Jones and Woodland, 1987), and that the ability (or competence) of the AC to respond to these signals is lost by stage 9.5. Using AC cells that have almost lost their competence, it was also shown that the vegetal pole actually has the capacity to induce mesoderm as early as the 32-cell stage (Jones and Woodland, 1987). Furthermore, it was shown that the type of mesoderm induced in the AC depended upon the region of vegetal pole to which it was conjugated, indicating some regional specification. Isolation of single blastomeres from the vegetal region of a 32-cell embryo and recombining with cells of the animal pole resulted in mesoderm induction in all cases. However, dorso-vegetal blastomeres (D1) induced dorsal-type mesoderm (somites and notochord), laterovegetal blastomeres (D2-4) induce intermediate mesoderm (muscle, mesothelium, mesenchyme, and blood) or ventral-type mesoderm (mesothelium, mesenchyme, and blood) (Dale and Slack, 1987). The finding that mesoderm is induced in the equatorial layer of the embryo by the vegetal region led to much work in the 80's and 90's to gain insight into the mechanism of induction

Upon MBT, VegT and other maternally localised factors accelerate zygotic transcription, which begins induction of mesodermal tissues (Kofron *et al.*, 1999). It is thought however, that the fate induced differs between the dorsal and ventral halves of the embryo as proven by the regional specification experiments already described (Dale and Slack, 1987). There is thought to be a pair of signals from vegetal to animal regions that qualitatively differ to result in formation of dorsal and ventral mesoderm (figure 1.6). This difference is thought to arise due to the synergistic action of both maternal factors and a dorsal modifier, thought to be dorsally localised β-catenin that has occurred in establishment of the D-V axis (Larabell *et al.*, 1997; Schneider *et al.*, 1996). The Nieuwkoop centre which is thus induced subsequently causes the formation of the gastrula signalling centre in the equator of the adjacent dorsal mesoderm, the Spemann's Organiser.

1.1.4.2 Mesodermal patterning is controlled by the Organiser

The Organiser was isolated in pioneering experiments by Spemann and Mangold in 1924 where it was showed that transplanting an explant from the dorsal lip of the embryo to the ventral side of another resulted in formation of a complete secondary axis. The Nieuwkoop centre itself continues to secrete mesoderm inducers and goes on to form the AE (Harland and Gerhart, 1997). Induction of the Organiser is thought to involve induction of the downstream Wnt targets, *Siamois* and *Xnr3* (Lemaire *et al.*, 1995; Smith *et al.*, 1995), in addition to the mesendodermal inducing signals likely to be the mesoderm inducing Xnrs (1,2,4,5,6). This results in the induction of Organiser specific markers such as *Goosecoid* (Gsc; Cho *et al.*, 1991). The Organiser acts at gastrula stages by releasing signals to adjacent cells, and its name stems from its ability to organise embryonic development (reviewed in De Robertis *et al.*, 2000; Harland and Gerhart, 1997). The third set of 'horizontal signals' are secreted by the Organiser and result in dorsalisation of the adjacent ventral mesoderm, neuralisation of the dorsal ectoderm, and anteriorisation of the endoderm. The Organiser therefore initiates specific tissue differentiation in the mesoderm, which ultimately is determined by its specific competence to respond to its signals (Harland and Gerhart, 1997). This is achieved via secretion of a plethora of antagonising factors, such as the Wnt antagonists *Dickkopf-1* (Glinka *et al.*, 1998) and *Frzb-1* (Leyns *et al.*, 1997) and the BMP antagonists *Noggin* (Smith *et al.*, 1993) and *Chordin* (Sasai *et al.*, 1994). From gastrula stages, the Organiser therefore subsequently establishes an area of low Wnt and BMP signalling by counteracting the ventralising BMP signals known to be expressed on the ventral side of the embryo. These ventralising signals are essential for ventral mesoderm formation (Kuroda *et al.*, 2004) and constitute a fourth signal in the model of mesoderm induction and patterning (figure 1.6).

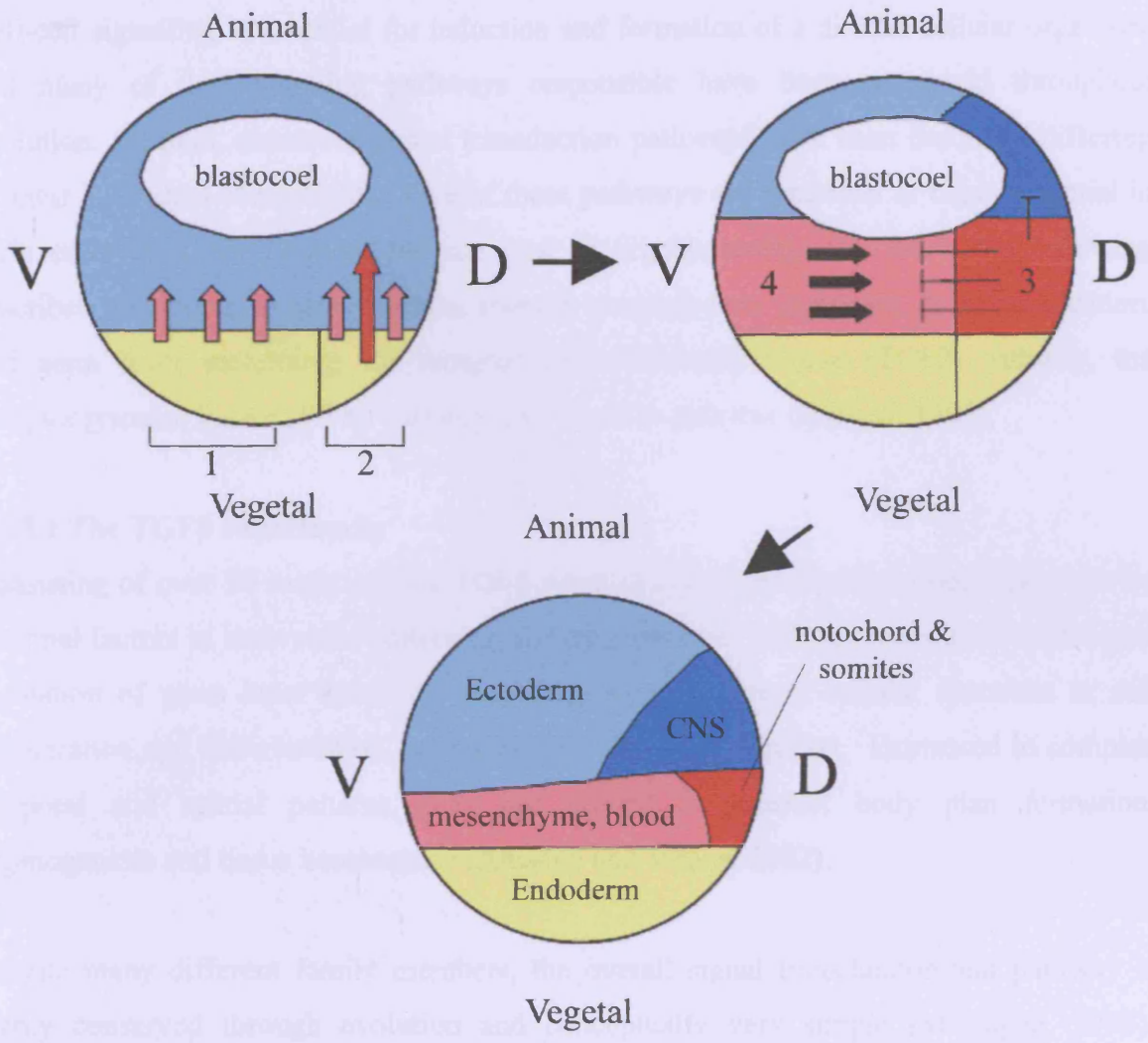


Figure 1.6 – The four signal model of mesoderm induction

Adapted from Wolpert (2007), mesoderm induction in *Xenopus* requires 4 distinct signalling events. The vegetal region secretes two signals **[1]** that induces ventral mesoderm, and a second **[2]** on the dorsal side of the embryo from the Nieuwkoop centre that initiates dorsal fate and induces the Organiser. The Organiser itself then secretes a third set of signals **[3]** that patterns and dorsalises the adjacent mesoderm, endoderm and ectoderm to form the dorsal mesoderm, anterior endoderm, and CNS respectively. This is achieved by inhibiting the fourth ventralising signals secreted from the ventral region **[4]**.

1.1.5 Intercellular Signalling in Embryonic Induction

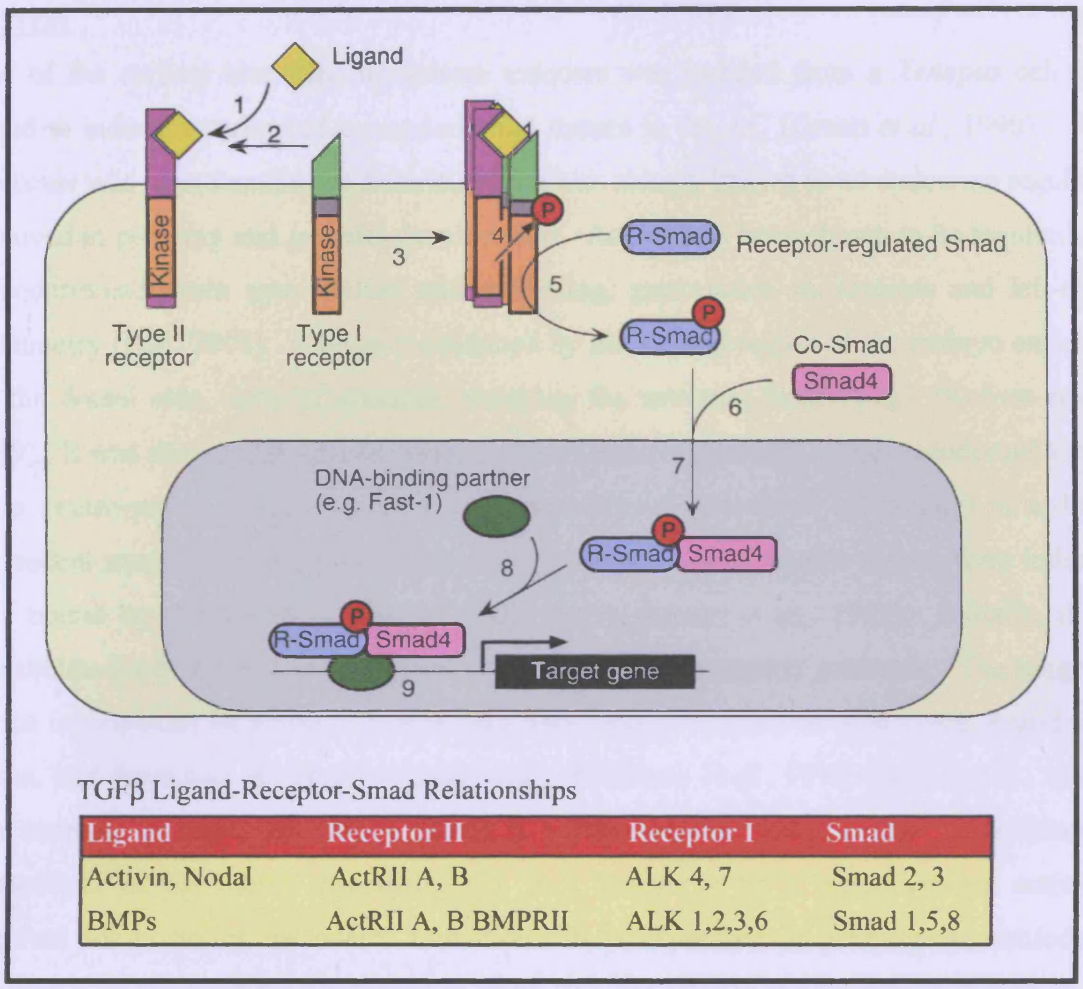
Cell-cell signalling is essential for induction and formation of a diverse cellular organism, and many of the signalling pathways responsible have been conserved throughout evolution. In total, seventeen signal transduction pathways have been described differing by their individual components. Five of these pathways are described as being essential in early embryonic development and are vital in organogenesis. Of these, the following described are shown to have essential roles in mesendoderm induction, axis specification, and germ layer patterning; the receptor serine/threonine kinase (TGFβ) pathway, the receptor tyrosine kinase (RTK) pathway, and the Wnt pathway (Gerhart, 1999).

1.1.5.1 The TGFβ superfamily

Consisting of over 30 members, the TGFβ superfamily of proteins have been shown to be essential factors in embryonic patterning and development, with key roles in formation and regulation of germ layer specification. They have important cellular functions in cell proliferation and differentiation, apoptosis, cell fate and migration. Expressed in complex temporal and spatial patterns, they are needed for correct body plan formation, organogenesis and tissue homeostasis (Attisano and Wrana, 2002).

Despite many different family members, the overall signal transduction and pathway is highly conserved through evolution and conceptually very simple (Massague, 1998). Ligands are bound by single pass transmembrane receptors with intracellular kinase domains, which are broadly divided into type I and type II receptors. Binding of extracellular ligands causes unidirectional phosphorylation of the type I receptor by type II, resulting in activation of its kinase domain. This results in phosphorylation and activation intracellular Regulatory Smads (RSmads; Smad1,-2,-3,-5, and -8). Activated RSmads then heterodimerise with the common Smad (coSmad4). The R-Smad/Smad4 complex can enter the nucleus due to the nuclear localisation signal of Smad4, and associate with various DNA binding proteins and transcriptional activators and repressors to control gene expression. Two further Smad proteins (Smad6 and 7) constitute the Inhibitory Smads (ISmads) that feedback to inhibit the receptor complex (figure 1.7; Attisano and Wrana, 2002; Massague, 1998).

Specificity between different TGFβ pathways comes at the level of the receptor. Seven different Type II receptors are generally shared between different members of the TGFβ family. However, different cellular responses are brought about by specific Type I receptors of which five have been identified (figure 1.7). The overall output however of all these ligands and receptors can be subdivided into two main groups; the Activin related family (including the Nodal related proteins [*Xnr1-6*], *Activin*, *Derriére,* and *Vg-1*) and the BMP subfamily (reviewed by Hill, 2001). Activin/nodal signalling is transduced through interaction specifically with the Activin receptor-Like Kinase (ALK) 4 and 7 (Reissmann *et al.*, 2001), whereas the BMP signalling is transduced through the remaining ALK receptors (reviewed in Dale and Jones, 1999). The activation of different receptors results in phosphorylation of different RSmads, with Smad2/3 specific to Activin/Nodal signalling and Smad1/5/8 to that of the BMP pathway (figure 1.7). Both pathways converge at coSmad4. The ability of different Smad complexes to drive unique gene expression (therefore correlating to different targets for BMP and Nodal signalling) is a highly regulated process. It is thought to involve fundamental differences in structure between the different Smad proteins that dictate their ability to bind DNA, and also the requirement for different transcriptional factor complexes. Careful regulation of the Smad proteins permits cell-type specific responses generating different cellular outcomes (reviewed by Ross and Hill, 2008). Therefore although inherently simple, the variety of permutations from receptor heterodimerisation and Smad complex formation and Smad interacting proteins gives rise to a very diverse range of cellular responses (reviewed in Derynck and Zhang, 2003).



TGFβ Ligand-Receptor-Smad Relationships

Ligand	Receptor II	Receptor I	Smad
Activin, Nodal	ActRII A, B	ALK 4, 7	Smad 2, 3
BMPs	ActRII A, B BMPRII	ALK 1,2,3,6	Smad 1,5,8

Figure 1.7 – The TGFβ signal transduction pathway

Adapted from Massague (1998; 2008). A schematic representing the conceptual signalling transduction pathway used by TGFβ proteins. Ligand binding to the type II receptor **[1]** in association with the type I receptor **[2]** forms the receptor complex **[3]**. The constitutively active Type II receptor phosphorylates conserved ser/thr residues on the kinase domain of type I **[4]**, which is then able to activate the appropriate Regulatory Smad (R-Smad, **[5]**). The active R-Smad heterodimerises with coSmad4 **[6]** resulting in nuclear localisation **[7]**. The Smad complex associates with a variety of co-transcriptional regulators **[8]** to drive gene transcription **[9]**. Both Activin and nodal show type II receptor promiscuity. The type I receptors are specific however, and result in pathway specific activation of different Smad proteins.

ACTIVIN

One of the earliest identified mesoderm inducers was isolated from a *Xenopus* cell line found to induce a variety of mesendodermal tissues in the AC (Green *et al.*, 1990). This molecule was later found to be Activin, which was already known as an endocrine regulator involved in pituitary and gonadal development. Activin has been shown to be required for mesoderm/endoderm specification and patterning, gastrulation movements and left-right asymmetry (Hill, 2001). Activin is produced by the vegetal region of the embryo enriched on the dorsal side, with its synthesis requiring the maternal factor VegT (Kofron *et al.*, 1999). It was shown that Activin was capable of inducing a range of mesendodermal tissue from ventro-posterior (mesenchymal) to antero-dorsal (notochord containing) in a dose-dependent manner. Furthermore, the levels at which these different tissues were induced was bound by distinct thresholds of concentration (Green *et al.*, 1992). Initially, these thresholds are broad and only become sharpened as development proceeds. The range of genes induced can be broadly divided into dorsal genes induced at high doses, non-dorsal genes, and those that are dorso-ventrally uniform (Green *et al.*, 1994; Symes *et al.*, 1994). For example, *Xenopus Brachyury* (*Xbra*) is a dorso-ventral uniform gene. The *Xenopus* homologue of the mouse *Brachyury (T),* it is known to be a transcriptional activator required for posterior mesoderm formation. It is expressed in presumptive mesoderm forming a ring around the blastopore at stage 10.5 and later in the notochord (Smith *et al.*, 1991). It is induced by moderate levels of Activin signalling. At higher doses, however expression of *Xbra* is inhibited (Green *et al.*, 1992). This was found to be the result of careful regulation achieved via feedback inhibition by the dorsal gene *Gsc,* a transcriptional repressor which is expressed at higher concentrations of Activin signalling preventing the expression of *Xbra* (Latinkic *et al.*, 1997). This dynamic action of Activin to produce a range of tissue at different concentrations provided evidence for morphogen gradients of signalling molecules in the embryo, whose non-uniform distribution differentially determines the fate a cell adopts (figure 1.8; reviewed by Green, 2002). Further evidence for the Activin as an endogenous inducer also comes from the finding that overexpression of a dominant-negative Activin receptor disrupted mesoderm formation in the embryo (Dyson and Gurdon, 1997; Hemmati-Brivanlou and Melton, 1992). Evidence for a direct role for Activin in mesoderm induction in *vivo* is doubted due to its low expression and the fact that blocking its expression in the embryo has contradictory effects on mesoderm specification. Overexpression of several truncated Activin receptors also blocked Vg1 mediated induction of mesoderm, whereas overexpression of the secreted protein Follistatin

(a specific antagonist of Activin) had no effect on mesoderm formation (Schulte-Merker *et al.*, 1994). A positive role was however reiterated by specific knockdown of Activin using MOs, where it was shown that interference with Activin signalling peturbed mesoderm formation in a dose-dependent manner (Piepenburg *et al.*, 2004).

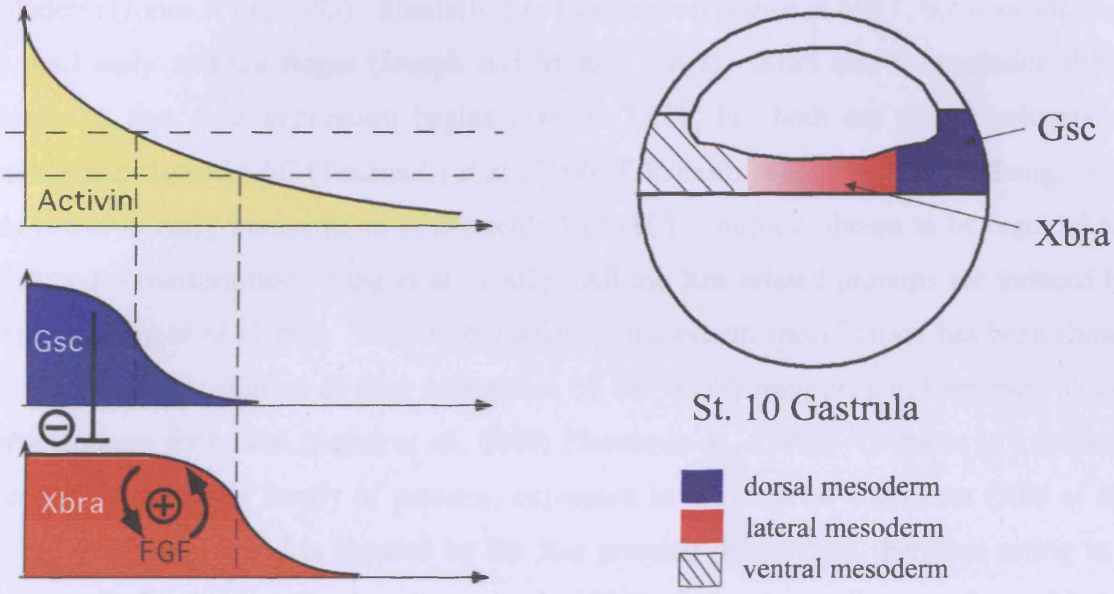


Figure 1.8 – Activin dose-dependent induction of Xbra and Gsc expression

Adapted from Green (2002), Activin induction demonstrates morphogen action. Moderate levels of Activin induce the pan-mesodermal gene *Xbra,* requiring FGF signalling to maintain its expression in a community effect. At high doses, Activin induces the dorsal gene *Gsc,* which induces dorsal mesoderm formation. In addition, it acts as a transcriptional repressor inhibiting *Xbra* expression. The result is a gradient along the D-V axis of different mesodermal tissues induced dependent upon the level of Activin signal received by the cells.

NODAL-RELATED PROTEINS

It has already been touched upon that the Nodal-related proteins have an important role in mesoderm induction, being expressed in the vegetal cells referred to as the Nieuwkoop centre (De Robertis and Kuroda, 2004). In *Xenopus* there are 6 Nodal homologues (Xnr1-6), with Xnr1,-2,-4,-5, and -6 shown to have potent mesoderm inducing capabilities (Jones *et al.*, 1995; Joseph and Melton, 1997; Takahashi *et al.*, 2000). Xnr1 and -2 expression begins at MBT and has been shown to dose-dependently induce markers of mesoderm and endoderm (Jones *et al.*, 1995). Similarly Xnr4 expression begins at MBT, but is maintained up until early neurula stages (Joseph and Melton, 1997). Xnr5 and 6 expression differ slightly in that their expression begins prior to MBT, but both are potent inducers of mesodermal tissue in AC (Takahashi et al., 2006; Takahashi et al., 2000). It is thought that this is due to early transcription of β-catenin-LEF-TCF complex, shown to be required for Xnr5 and 6 transcription (Yang et al., 2002). All the Xnr related proteins are induced by VegT (Kofron *et al.*, 1999). Their importance for mesoderm specification has been shown by the fact that inhibition of their expression by the endodermal protein Cerberus, blocks mesendoderm formation (Agius *et al.*, 2000; Piccolo *et al.*, 1999). Cerberus is a secreted member of the DAN family of proteins, expressed in the anterior endoderm (AE) of the gastrulating embryo and is induced by the Xnr proteins themselves, therefore acting in a negative feedback loop (Bouwmeester *et al.*, 1996). It has been shown to block Nodal, BMP, and Wnt signalling (Piccolo *et al.*, 1999). It has already been described that the maternally localised VegT and β-catenin is responsible for inducing Xnr expression (Kofron *et al.*, 1999), positioning them as early targets for mesendoderm specification and embryonic patterning at MBT. Subsequent work by the De Robertis lab showed a gradient of Nodal signalling exists from dorsal-ventral and is vital for correct patterning of the embryo (Agius *et al.*, 2000). This was achieved using a truncated version of *Cerberus* (*CerS*) which specifically blocks the Nodal pathway (Piccolo *et al.*, 1999). It was shown that *Xnr* expression is blocked by *CerS* (with exception of Xnr3) and is required for Organiser formation. Furthermore, all Xnrs were found to be enriched in the dorsal regions of the vegetal pole and could induce mesoderm in AC in a dose-dependent manner, with low levels inducing markers of ventral mesoderm (*Wnt8)* and higher levels inducing more dorsal mesoderm (*Gsc*). This was concluded to be in a similar manner to that described for Activin, and recapitulates the D-V patterning that occurs in the embryo (Agius *et al.*, 2000). This was further shown by analysis of the activated form of intracellular mediator of Nodal signalling, Smad2. Using an antibody against phosphorylated Smad2, it was shown that

Smad2 activation occurs after MBT enriched on the dorsal side of the embryo (correlating with the Nieuwkoop centre and β-catenin localisation). Smad2 activation then moves in a wave from dorsal to vegetal regions, but its dorsal expression begins to be attenuated by expression of antagonists induced by Xnr expression, namely *Cerberus* (figure 1.9; Lee et al., 2001). This strongly implicates members of the TGFβ family as key regulators in formation and patterning of the mesoderm and endoderm (Tian and Meng, 2006).

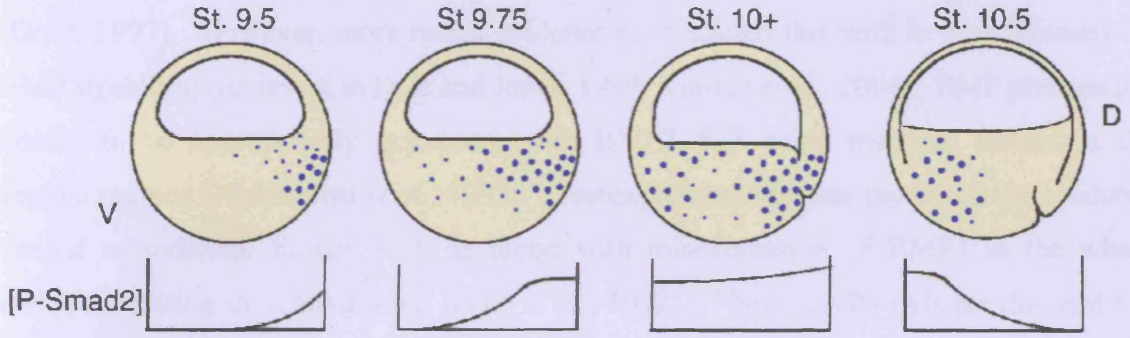


Figure 1.9 – Dorsal to ventral Nodal expression progresses in a wave of activation

Taken from Green (2002), active nodal signalling as determined by nuclear staining of phosphorylated Smad 2 (blue dots) in sagittal sections of late blastula/early gastrula embryos. Graphs depict phospho-Smad2 distribution throughout the embryo. Overall, Smad2 activation progresses from the dorsal side of the embryo, where it is induced by active nodal signalling by the Nieuwkoop centre. This then progresses to the ventral side of the embryo, with signalling in the dorsal half attenuated by expression of the Nodal antagonists *Antivin* and *Cerberus*.

BONE MORPHOGENETIC PROTEINS

The Bone Morphogenetic Proteins (BMPs) are a family of proteins belonging to the TGF-β superfamily, with 5 family members in *Xenopus* showing homology to their mammalian counterparts (Dale and Jones, 1999). The initial model for mesoderm induction was a 3 signal model, as it was thought that ventral mesoderm was a default state of the embryo (Slack, 1994). Extirpation and culture of regions of ventral tissue showed unpatterned cells expressing ventral mesodermal markers of blood and mesenchyme. Similarly, UV treatment of embryos causes a lack of all dorsal tissues and only the presence of ventral derivatives. It was therefore believed that ventral fates were the inactivated ground state (Graff, 1997). However, more recent evidence contradicted this with key involvement of BMP signalling (reviewed in Dale and Jones, 1999; Kuroda *et al.*, 2004). BMP proteins are known to be appropriately expressed, with BMP2,-4,-7 being maternal factors in the vegetal regions (Nishimatsu *et al.*, 1992). Overexpression of these proteins in AC induces ventral mesodermal tissues such as blood with misexpression of BMP4 in the whole embryo resulting in ventralisation (Dale *et al.*, 1992). These results indicate that BMP is capable of conferring ventral character. Their role in *vivo* was re-iterated by the finding that blocking BMP signalling using a truncated form of the BMP receptor lacking the tyrosine kinase domain results in conversion to dorsal mesoderm, suggesting ventral mesoderm needs to be induced and requires BMP signalling (Graff *et al.*, 1994). In the embryo early maternal BMP2/4/7 expression at blastula stages is uniform, but as development proceeds their expression is restricted from the dorsal regions by the Organiser (reviewed in Dale and Jones, 1999). This is due to the secretion of the extracellular BMP antagonists, *Noggin* and *Chordin* in addition to endodermal expression of *Cerberus*. Thus a graded expression of BMPs is apparent in the gastrula embryo from ventral to dorsal, and dorsal and ventral signalling centres oppose each other to pattern the mesoderm for ventral tissue induction (figure 1.10) Active BMP signalling therefore constitutes a 4^{th} signal in mesoderm induction to induce ventral tissue and oppose dorsal influence of the Organiser. Loss of its expression results in the formation of dorsal mesoderm that is normally overridden in ventral regions by BMP signalling (Graff, 1997; Kuroda *et al.*, 2004).

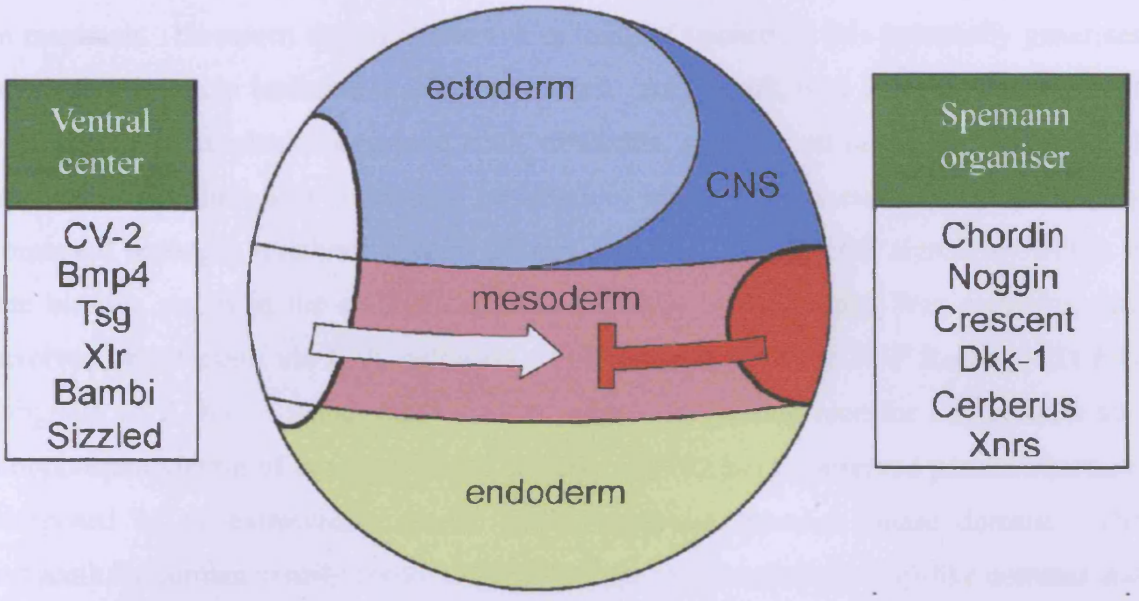


Figure 1.10 – Mesodermal patterning depends on opposing signals from dorsal and ventral signalling centres

Adapted from De Robertis & Kuroda (2004), the gastrula embryo has two distinct domains that secrete dorsalising and ventralising factors. Spemann's Organiser on the dorsal side secretes a variety of BMP antagonists that dorsalises the surrounding mesoderm by inhibiting signals secreted by the ventral signalling centre.

1.1.5.2 Fibroblast Growth Factor family

Fibroblast Growth Factors (FGFs) are a large family of proteins with currently 22 members in mammals. However, due to alternative splicing of transcripts this potentially generates hundreds of protein isoforms in different tissues. As a result, they have an abundance of roles in the developing embryo and adult vertebrate, such as cell migration, cell survival and apoptosis, limb axis formation, neurulation, and organogenesis. FGFs are highly conserved amongst vertebrate species (Ornitz and Itoh, 2001). FGF signalling begins at late blastula stages in the embryo regulated carefully by Nodal and Wnt pathways, and involves transduction via RTK pathways. FGF proteins bind the FGF Receptor (FGFR) subgroup of RTKs of which there are four members, causing receptor dimerisation and autophosphorylation of the intracellular domain. FGFRs have conserved protein structure, composed of an extracellular region and intracellular tyrosine kinase domain. The extracellular domain usually consists of two or three immunoglobulin (Ig)-like domains and a heparin sulphate binding domain, vital for ligand binding (Ornitz, 2000). The extracellular domain is alternatively spliced generating different receptors with varying ligand specificity and affinity. For example, alternative splicing in the third Ig domain can generate two different isoforms (IIIb or IIIc) which have very different ligand specificity. This process is determined in a tissue-specific manner (Zhang *et al.*, 2006). Such mechanisms generate a diverse range of isoforms of only a small number of genes, required for the large range of cellular functions, ligands, and mechanisms of action of the FGF signalling pathway (Ornitz, 2000). Signal transduction then most commonly occurs via the *Ras/MAPK* pathway, but also via the PLCγ or PI_3 kinase pathways (figure 1.11; Bottcher and Niehrs, 2005).

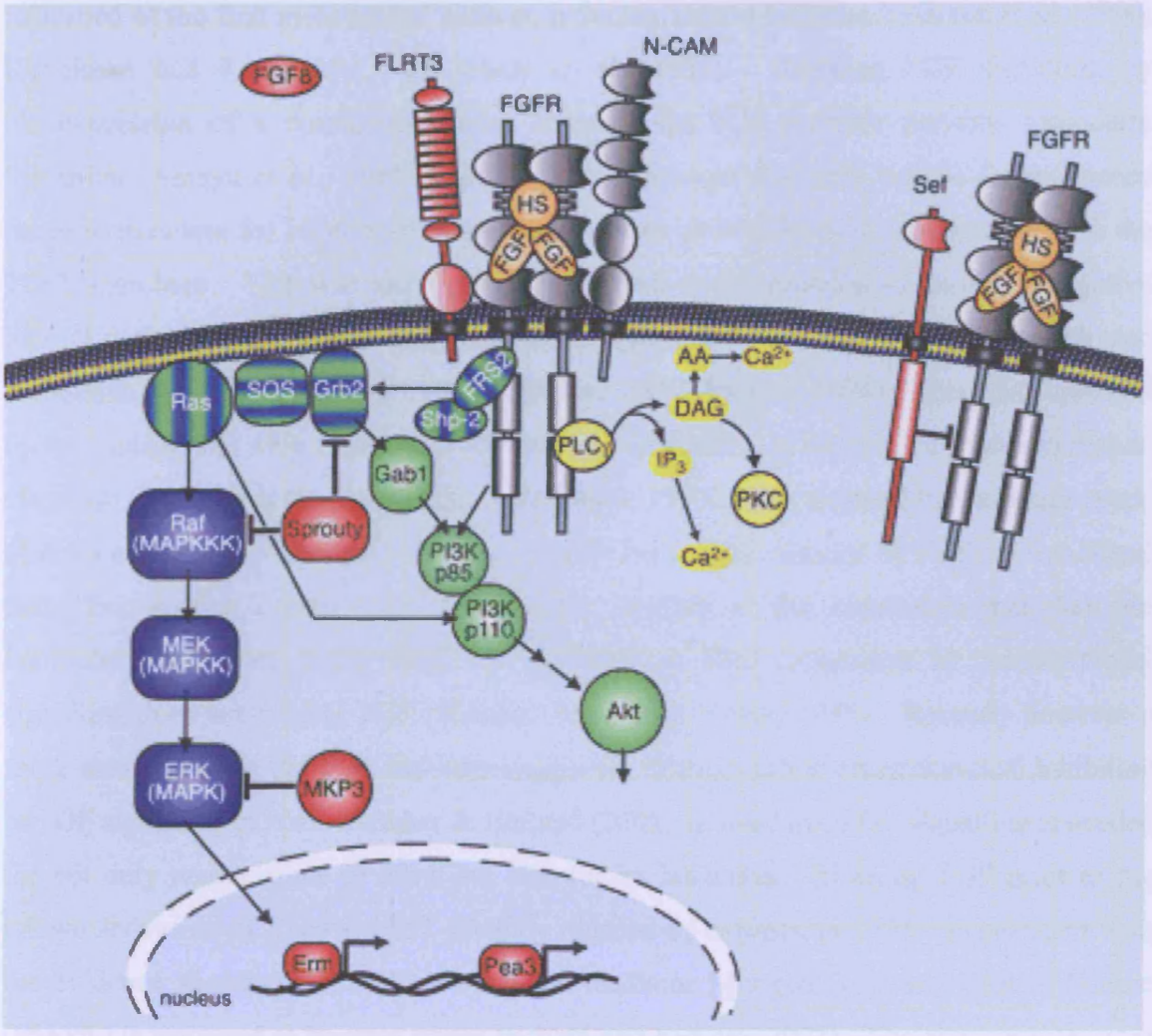


Figure 1.11 – The Fibroblast Growth Factor transduction pathway

Taken from Böttcher & Niehrs (2005). FGF proteins bind to their receptor causing dimerisation and subsequent autophosphorylation, which activates intracellular cascades. FGF signal transduction occurs most commonly via the **MAPK** pathway, but also via the **PLCγ/Ca^{2+}** or **PI_3 kinase/Akt** pathways. The MAPK pathway involves the lipid anchored protein FRS2, which is phosphorylated on ligand binding forming a protein complex that activates Ras. This results in activation of the MAPK cascade, which eventually leads to phosphorylation of ERK by MEK. Phospho-ERK is then capable of entering the nucleus to activate specific transcription factors that can drive gene expression

It has been shown that FGF is capable of inducing mesodermal tissue with FGF2 being identified as the first mesodermal inducer, inducing ventral mesoderm (Green *et al.*, 1990; Kimelman and Kirschner, 1987; Slack *et al.*, 1987). Blocking FGF signalling by overexpression of a dominant-negative form of the FGF receptor prevents mesoderm formation (Amaya *et al.*, 1991). However, it is thought that FGF acts as a competence factor to maintain the identity of induced mesoderm, potentially acting in synergy with the TGFβ members. This was shown by the fact that overexpression of dominant-negative FGFR1 was shown to block Activin-mediated mesoderm induction in AC (Cornell and Kimelman, 1994; Cornell *et al.*, 1995; LaBonne and Whitman, 1994). This was supported by the finding that *Xbra* expression requires FGF signalling to maintain its own expression via positive feedback (Schulte-Merker and Smith, 1995). It is induced by moderate levels of Activin/Nodal signalling (Green *et al.*, 1992), but is also induced by FGF. It was found that *Xbra* is also capable of inducing FGF, leading to the conclusion that they are components of a regulatory loop, but initiation of *Xbra* expression by Activin/Nodal signalling does not require FGF (Schulte-Merker and Smith, 1995). Recently however a more instructive role for FGF has been suggested, through timed pharmaceutical inhibition of FGF signalling in *vivo*. Fletcher & Harland (2008) showed that FGF signalling is needed for not only maintenance of *Xbra* but also for its induction. Blocking FGF prior to the known activation of *Xbra* at MBT severely reduced its expression. This is consistent with the evidence showing the FGF downstream mediator Mitogen Activated Protein Kinase (MAPK) is activated at blastula stages (Schohl and Fagotto, 2002). The exact involvement of FGF as a mesoderm inducer is therefore still not fully understood.

1.1.5.3 The Wnt signalling network

Wnts are a large family of secreted cysteine-rich glycoproteins that signal through seven transmembrane receptors of the Frizzled (Frz) family. Wnt proteins are involved in a wide range of developmental processes, including anterior-posterior patterning, axis formation and gastrulation, neural development and maintenance, and joint formation. Hence, defects in Wnt signalling result in a variety of different embryonic and developmental defects, as well as a variety of disease (reviewed by Logan and Nusse, 2004). Wnt signal transduction is a complex process involving many extracellular, cytoplasmic and nuclear regulators, and until recently thought to act in three distinct pathways; Wnt/β-catenin, Wnt/Calcium, and Wnt/JNK pathways (Kestler and Kuhl, 2008)

In the case of canonical signalling, Wnt proteins bind Frizzled receptor and the signal is transduced by Dsh, ultimately preventing phosphorylation that normally results in degradation of the transcriptional regulator β-catenin. In the nucleus, β-catenin acts as an essential co-activator for Lymphoid Enhancer-binding Factor/T Cell specific transcription Factor (LEF/TCF) DNA binding transcription factors (Logan and Nusse, 2004). In absence of ligand, β-catenin is degraded by formation of a destruction complex consisting of GSKβ resulting in phosphorylation and degradation by the proteasome. Binding of ligand to Frz and co-receptor low density lipoprotein receptor-related protein-5/6 (LRP) leads to stabilisation and nuclear localisation of β-catenin by disassembly of the destruction complex. The non-canonical pathway involves G-proteins, phospholipase C (PLC), protein kinase C (PKC), Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII), c-jun kinase (JNK), and Rho GTPases, and is often divided into Wnt/Ca^{2+} and Wnt/Planar Cell Polarity (PCP) pathways (Eisenberg and Eisenberg, 2006). In addition, several families of Wnt antagonists exist that have important roles in regulating activation of the Wnt pathway; the secreted Frizzled-related protein (sFRP) class directly bind Wnt ligands and include *Cerberus* whereas the Dickkopf class bind to the LRP co-receptor (reviewed by Kawano and Kypta, 2003).

Recent evidence has suggested that this simplistic view of defined Wnt pathways is inaccurate. For example, previously ligands belonging to the Wnt pathways were divided into the canonical Wnt1 group (including Wnt1/3a/8), and non-canonical Wnt5 group (Wnt4/5a/11; Eisenberg and Eisenberg, 2006). However, it has been shown that several members can activate more than one pathway. Wnt11 was previously described as a non-canonical Wnt ligand. Recent evidence however has suggested it can also influence the canonical Wnt pathway, shown to both activate the canonical Wnt pathway during axis specification (Tao *et al.*, 2005) and inhibit it at later developmental stages (Maye *et al.*, 2004; Torres *et al.*, 1996; reviewed by Weidinger and Moon, 2003). In addition, it has been shown that the previously determined canonical Wnt inhibitor *dkk-1* can also activate the non-canonical pathway (Pandur *et al.*, 2002). The three linear pathways previously described are therefore not as clearly separated as initially thought. As a result, where previously it was thought that pathway specificity was determined by ligand or Frz receptor it is now believed specificity is governed by the vast permutations of Wnt ligand/receptor/co-receptor/intracellular component complexes that are possible. It has

hence been more recently classified as a Wnt signalling network (reviewed in Kestler and Kuhl, 2008).

The importance of Wnt signalling in early development has already been described. Members of the Wnt signalling pathway are known to be maternally localised (Larabell *et al.*, 1997; Weaver and Kimelman, 2004) and result in dorsal localisation of β-catenin important in D-V axis specification (Heasman *et al.*, 1994; Molenaar *et al.*, 1996). Also, in early germ layer formation Wnt signalling is known to be required for posterior mesoderm formation (Christian and Moon, 1993). Wnt8 expression begins in the late blastula exclusively in the ventral mesoderm. Overexpression of Wnt8 from MBT under the control of the cytoskeletal actin (CSKA) promoter enhances formation of posterior structures such as the blood islands at the expense of dorsal structures (Christian and Moon, 1993). However, overexpression of Wnt8 in AC does not induce mesoderm. It was found however, that Wnt8 can modify the type of mesoderm induced by FGF and TGFβ. Normally FGF induces ventral mesoderm in AC (Green and Smith, 1990), but co-injection with Wnt8 alternatively induced dorsal mesoderm such as muscle and neural tissue (Christian *et al.*, 1992). Similar evidence for Activin response in AC also exists (Sokol and Melton, 1992). In addition, several Wnt antagonists such as FrzB, Dkk-1 and crescent, are secreted by the Organiser and are required to create an area of low Wnt signalling necessary for head development and dorsalisation (Glinka *et al.*, 1998; Glinka *et al.*, 1997; Piccolo *et al.*, 1999). Ectopic expression of Wnt antagonists results in dorsoanteriorisation, with expansion of cement glands and inhibition of posterior development (Leyns *et al.*, 1997). There is therefore strong evidence to suggest involvement of Wnt signalling in mesodermal induction and patterning, with a balance between its activation and antagonism required for formation of ventral and dorsal tissue, respectively (Kuroda *et al.*, 2004)

1.1.6 Specification of different tissues within a Germ Layer

Formation of the germ layers is therefore a complex process involving interactions of many different signalling pathways in an organised manner. Generation of the primary germ layers is only the beginning of overall embryonic patterning. For example, the mesoderm, ectoderm and endoderm in close cellular proximity to the Organiser are exposed to a

similar range and concentration of signals. However, cells of the different territories have different responses and develop into distinct cell types; ectoderm forms neural tissue, mesoderm forms somites, endoderm forms anterior gut (Gerhart, 1999). Furthermore, each territory can give rise to a range of different cell types, and one of the challenges in studying embryonic patterning is understanding how individual organs develop from within these broad germ layers. Individual organs themselves (with exceptions) can often be described as groups of cells located in developmental fields, which are homogenous populations before they differentiate (Thisse and Zon, 2002). As already shown, the mesodermal germ layer gives rise to distinct cellular types along the dorsal-ventral axis (Dale and Slack, 1987), with formation of dorsal mesoderm the result of dorsalising signals from the Organiser. There is therefore a careful balance between action of dorsalising and ventralising signals, and the overall character induced upon the mesoderm depends upon the overall sum of these two forces (De Robertis and Kuroda, 2004). However, what is less well-characterised is the exact mechanism by which regions intermediate along the D-V axis adopt a particular fate (so-called intermediate mesoderm). Several suggestions exist as to the origins of such tissue. One line of thought is that different cells are merely responding differently as result of their position along a morphogenetic gradient (Green, 2002), and it is their interpretation of this gradient that generates a different cellular response. It is likely however, that this interpretation is further complicated by interaction of many different signalling events, and not merely relying solely on the balance of dorsalising and ventralising signals it receives (Harland and Gerhart, 1997). One such example is that of the heart. The heart forms from precardiac mesoderm that originates at an intermediate layer within the mesoderm of the gastrulating embryo (figure 1.12). However, the precise mechanisms by which it is induced have not been fully elucidated.

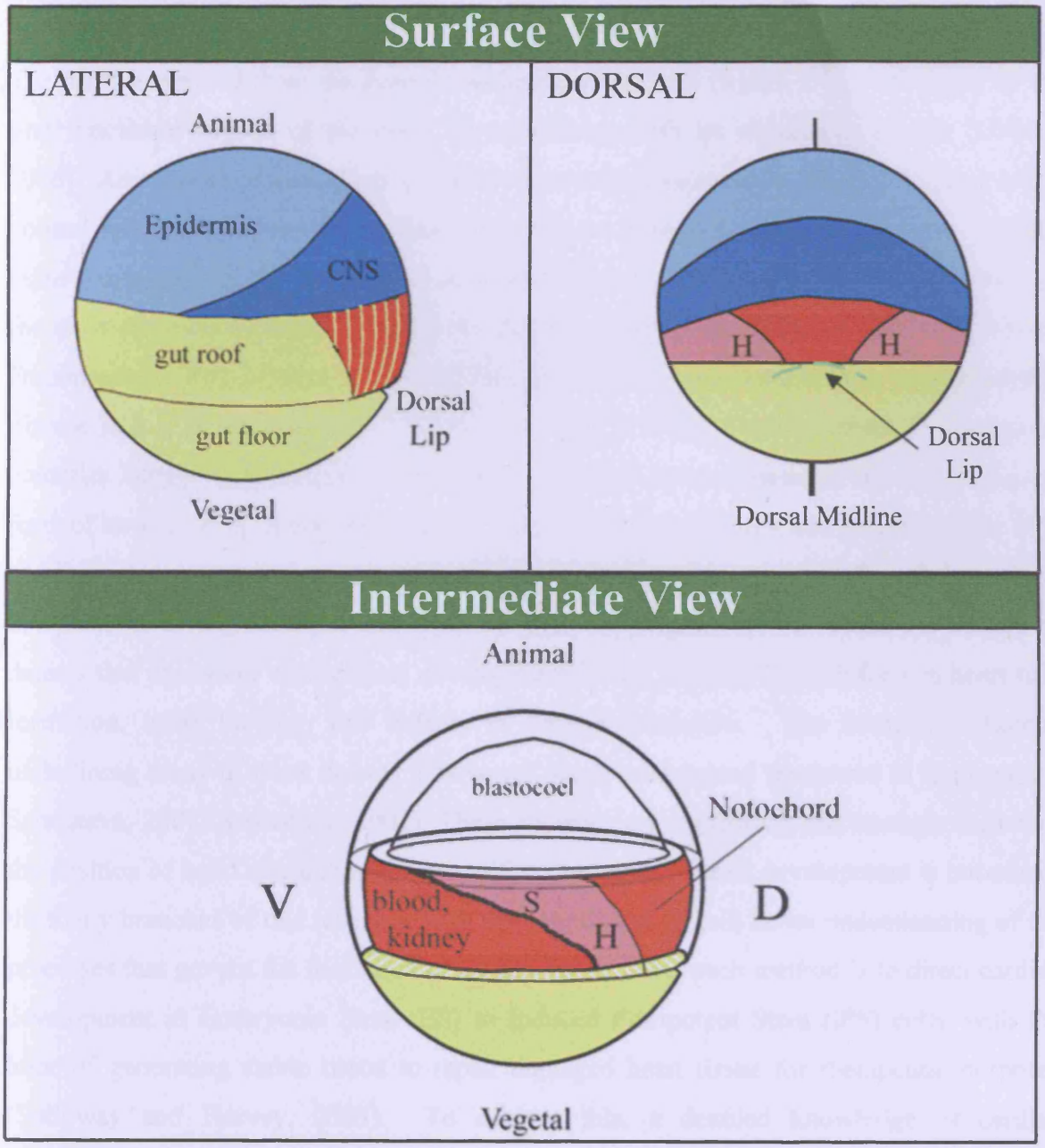


Figure 1.12 – Cardiac tissue develops from the intermediate mesoderm

Adapted from Harland & Gerhart (1997), various layers of the *Xenopus* gastrula are shown. Adjacent to the dorsal lip, the Organiser is induced and patterns the mesoderm (red cross-hatching). The heart is one of many tissues derived from the mesoderm. The cardiac precursors arise adjacent to the dorsal lip in close contact with the Organiser. Heart tissue (H) is derived from a region of intermediate mesoderm, as are the somites (S), whereas ventral mesodermal tissues include kidney and blood. The induction of such intermediate mesodermal derivatives is relatively poorly described.

1.2 Formation of the Cardiac Tissue

The heart is derived from the deep dorso-lateral mesoderm (figure 1.13) and is one of the first functional organs of the body, in association with its circulatory system (Gilbert, 2006). Any abnormalities affecting itself or its vasculature result in severe disruption to the normal functioning of the body. The range of heart defects and disorders is vast. Cardiac failure, the result of any structural or functional disorder that impairs its ability, is amongst the most common causes of death in the Western World. According to the World Health Organisation, 30% of total deaths worldwide in 2003 were attributed to cardiovascular disease and it is predicted by 2010 it will be the leading cause of death in developing countries (http://www.who.int). Similarly congenital heart disease is the most frequent form of birth defects, occurring in around 2% of newborn children and accounting for 25% of all human congenital abnormalities (Nemer, 2008). The process of cardiogenesis is complex, involving multiple cell types of different origins. As a result, the variety of defects that can occur during heart development is vast, ranging from defects in heart tube formation, heart looping, and defects in septum formation. The complex aetiology underlining many of these defects is however poorly understood (reviewed in Ransom and Srivastava, 2007; Srivastava, 2001). These statistics are significant and strongly influence the position of heart research in the scientific community. Heart development is but one of the many branches of this research, with the overall aim to gain better understanding of the processes that govern the formation of cardiac tissue. One such method is to direct cardiac development in Embryonic Stem (ES) or Induced Pluripotent Stem (iPS) cells, with the hope of generating stable tissue to repair damaged heart tissue for therapeutic purposes (Solloway and Harvey, 2003). To achieve this, a detailed knowledge of cardiac specification is thus required.

1.2.1 The Vertebrate Heart – the Process of Cardiogenesis

The vertebrate heart is complex structure consisting of atrial and ventricular chambers. Its development involves a variety of morphogenetic changes that give rise to the sophisticated, muscular structure that provides the efficient pumping system of the adult heart. As with much of early development, cardiogenesis is a regulated step-wise process.

It begins with specification and recruitment of cells to the cardiac lineage, migration and fusion of early progenitor cells and subsequent structural changes, or morphogenesis. This result in the generation of a variety of defined inter-connected muscles of the adult heart, which is networked to the body via its associated vasculature (reviewed by Harvey, 2002). At present, the degree of understanding is not uniform; the morphogenetic events and how they are regulated are relatively well understood (although not at the molecular level), the differentiation process is known to a lesser extent, but with regards to initial induction and recruitment it is poorly defined. Cardiogenesis will therefore be discussed from the reverse order of its development, from morphogenesis and tissue differentiation to specification of cardiac precursors

1.2.1.1 Evolution of cardiac development

Throughout evolution, heart morphology has developed extensively. However despite the diversity of overall body plans between different species, the genetic programs that lead to heart formation have been very well conserved (reviewed by Olson, 2006). The fundamental heart unit is composed of the cardiac muscle cells (actin, myosin, tropomyosin), believed to have originally arisen as an ancient means of fluid movement during feeding. These have evolved into cardiac cells that have further developed into the cardiomyocytes of the modern heart. The first vessel is believed to have been a linear peristaltic tube, similar to that of the *Drosophila* dorsal vessel. This evolved into a more powerful chambered pump that showed synchrony, linked to a closed circulatory system. The final evolutionary hallmark in vertebrates arose from development from aquatic to terrestrial forms which required separation of oxygenated and de-oxygenated blood. There are obvious morphological differences accompanying evolution of the heart tube. These include features such as asymmetry, differences in looping, and overall size. Such differences are the result of differing requirements upon the heart in different organisms. For example, vertebrate heart tubes are distinctly bigger composed of thicker muscle and continuous endothelial lining. Such requirements are due to the need to generate higher pressures compared to their evolutionary ancestors (reviewed in Fishman and Chien, 1997).

The ancestral transcriptional network involved in heart formation is governed by a core of transcription factors, which also regulate themselves to reinforce the cardiac program. The MADS box protein MEF2 is the most ancient factor associated with differentiation of all muscle types (Olson, 2006). Many genetic pathways are conserved through evolution but

the increasing structural complexity occurred through expansion of the regulatory networks responsible for its formation (figure 1.13).

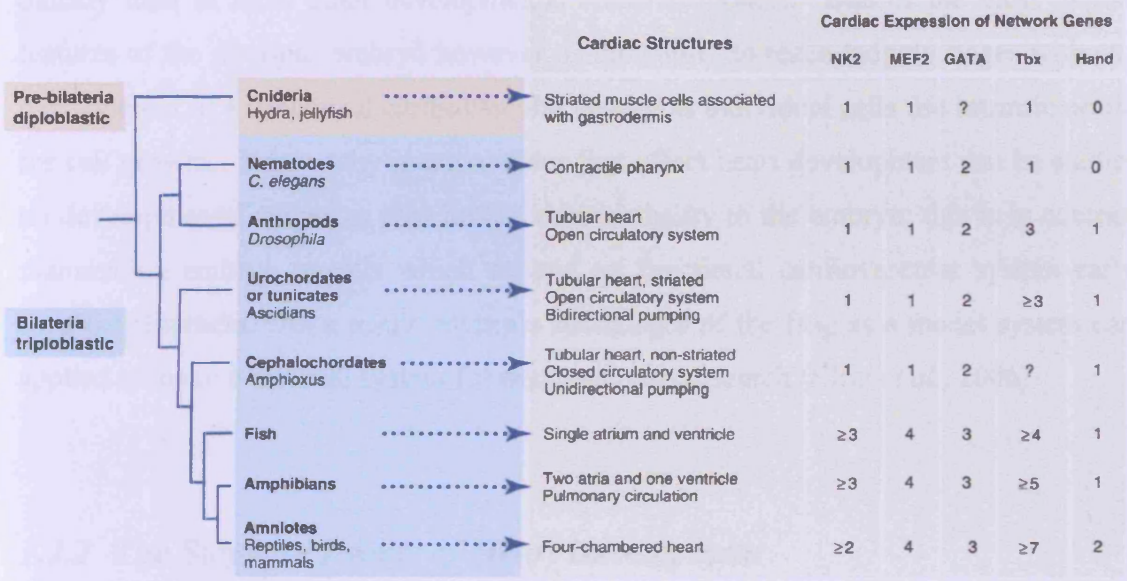


Figure 1.13 – Evolutionary conserved network of cardiac transcription factors

Taken from Olson (2006), the heart has evolved from a simple peristaltic tube to a complex multi-chambered organ. Through evolution, the complex networks involved have grown, with duplications increasing the number of genes, leading to increased number of proteins reflected in increased complexity. Despite obvious anatomical differences amongst vertebrates, many of the key regulatory networks are conserved.

2.2.1.2 The amphibian as a model for vertebrate cardiogenesis

A major focus of criticism for the use of *Xenopus* as a cardiac model is due to obvious anatomical differences of its adult heart in comparison with other vertebrates. This is partly the result of its adaptation to its aquatic environment. The most notable of these includes a three chambered heart of two atria and one highly trabeculated ventricle. In addition, the heart has cranially positioned arterial and venous connections and distinct valve differences that permit control of blood flow dependant upon resistance in vascular beds (Blitz *et al.*, 2006; Lohr and Yost, 2000). Despite the existence of large differences in gastrulation between different vertebrates, many key cardiac network genes are conserved (figure 1.13). Many early developmental decisions are identical to other vertebrate models, and thus

precursor migration, tube formation and looping are well studied (reviewed by Fishman and Chien, 1997). Thus, despite these obvious anatomical differences *Xenopus* provides a useful model for vertebrate cardiogenesis (reviewed by Warkman and Krieg, 2007). Furthermore, formation of the heart and other organs in the amphibian occurs much more quickly than in most other developmental research models. One of the most important features of the *Xenopus* embryo however, is the ability to reach tadpole stages without the development of a functional cardiovascular system as individual cells use intrinsic nutrients for cell growth. Hence, any manipulations that affect heart development can be studied at all developmental stages as they do not cause lethality to the embryo; this is in contrast to mammalian embryo models which depend on functional cardiovascular system early in their development. As a result, multiple advantages of the frog as a model system can be applied to make it an ideal system for organogenesis research (Blitz *et al.*, 2006).

1.2.2 The Stepwise Events of Heart Development

As discussed, the process of cardiogenesis is carefully regulated and can only occur once cell fate is specified. Only then may differentiation, the expression of functional proteins specific to an organ, occur. This is followed by the subsequent morphogenetic events driven by these proteins (Nascone and Mercola, 1996). It is therefore obvious to note that these events all take place at specific time points through embryogenesis specific to the particular species, with the relative time-course of *Xenopus* shown in Table 1.1.

Stage of Cardiogenesis		Developmental Stage	
1. Heart Field Specification	Initiation Cardiac Program	Stage 10-13	Gastrulation, Nkx2.5 expression
	Migration of Precursors	Stage 26-28	Tailbud, tropomyosin deposition
2. Early Differentiation	Heart Tube Formation	Stage 31-33	Tailbud
3. Morphogenesis	Contraction	Stage 35	Tadpole
	Looping	Stage 33-36	Tadpole
	Adult Heart	Stage 46	Tadpole

Table 1.1 – Cardiac Developmental Stages in *Xenopus laevis*

Adapted from Lohr & Yost (2000), the stages of cardiac development in *Xenopus* occur at distinct time periods, and are characterised by the use of cardiac markers distinct for each stage. Developmental stages are those defined by Nieuwkoop & Faber (1994). During specification, the cardiac mesoderm generates a field of competence termed the 'heart field' marked by *Nkx2.5* expression (stage 10-13). Subsequent cardiac gene expression of myosin, actin, and troponin isoforms results driving morphogenesis, and subsequent adult heart formation (stage 26-28).

1.2.2.1 Origin of cardiac precursors

It is thought that cardiac specification occurs early in the gastrulating embryo. Major evidence for the initiation of cardiac specification came from experiments by Sater & Jacobson (1989), who studied heart specification in explants of prospective heart mesoderm by assaying their ability to self-differentiate in *vitro*. Isolation of explants at mid-neurula stages were shown to form beating tissue in nearly 100% of cases. However, extirpation of late gastrula pre-cardiac mesoderm only formed beating tissue in 40% of cases, the incidence of which was increased upon inclusion of underlying endoderm (Sater and Jacobson, 1989). Subsequent work in the Jacobson lab went on to further characterise the

early timing of cardiac specification. It was found that 90% of explants formed beating hearts when extirpated at stage 10.5, and concluded that heart specification is complete by late gastrula stages prior to stage 12.5 but no earlier than stage 10 (Sater and Jacobson, 1990b).

In addition in this study, the origins of the cardiac precursors were examined (Sater and Jacobson, 1990b). Followed through explant experiments of the dorsal lip region of the early embryo (which demarcates the Organiser), cardiac precursors are found to originate as symmetric patches of dorso-lateral mesoderm within 45° of the dorsal midline (figure 1.14). This therefore demonstrated that heart precursors do not originate in the final ventral region in which the heart resides (Sater and Jacobson, 1990b). Their experiments compared specification of different regions of Marginal Zone (MZ; regions of both endoderm and mesoderm), and found that only those containing the dorsal lip develop heart. They concluded that the first step of heart formation is establishing the D-V pattern of the precardiac mesoderm, resulting from signals from the dorsal lip. The location of these cardiac precursors was further confirmed by work of Mercola and colleagues (Nascone and Mercola, 1995).

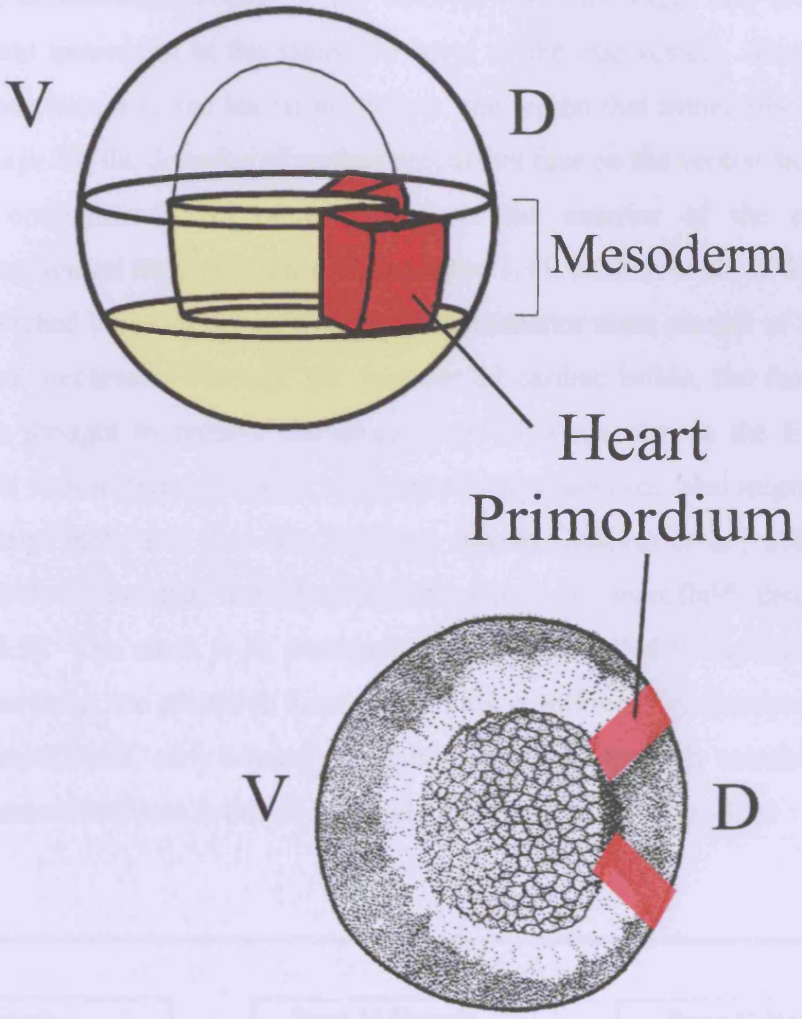


Figure 1.14 – Origins of the cardiac primordia

Adapted from Foley *et al.* (Foley *et al.*, 2006). Based upon extirpation experiments of the Dorsal Marginal Zone, cardiac precursors arise in asymmetric patches adjacent to the dorsal lip and Spemann's Organiser.

Gastrulation, a process of extensive cell movements and rearrangement that gives rise to the final position of the tissues of the embryo, results in migration of cardiac precursors to the presumptive dorsoanterior region of the embryo. By this stage, they are located in the anterior lateral mesoderm at the same A-P level as the otic vesicle. During neurulation, migration then occurs to the lateral mesoderm, the region that eventually gives rise to the heart. By stage 28, the domains of cardiac precursors fuse on the ventral side of the embryo forming a contiguous sheet of mesoderm at the anterior of the embryo initially encompassing ventral tissue including liver (figure 1.15; Mohun et al., 2003). This forms a collar sandwiched between the cement gland (the anterior most marker of the embryo) and ventral blood precursors. Through the example of cardiac bifida, the fusion of the heart primordia is thought to involve the distribution of fibronectin in the ECM, as its loss prevents their fusion (Harvey, 2002). Some precursors however, also migrate further to the posterior lateral plate and give rise to blood islands (Marvin *et al.*, 2001). This is an example of a developmental restrictive field known as the heart field (discussed further in section 1.2.2.3). The result is an endothelial tissue surrounded by myocardial epithelium, otherwise known as the primitive heart tube. This is followed by terminal differentiation. As already mentioned, early events are highly conserved through vertebrates with many similarities shared between avian, mouse and amphibian research models.

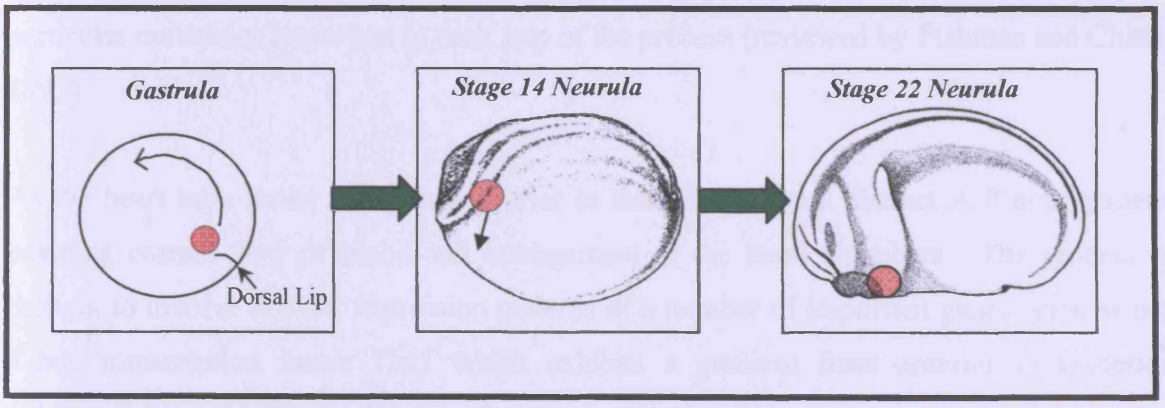


Figure 1.15: Migration of Early Cardiac Precursors

Adapted from Mohun *et al.* (2003), a schematic of the origins and migratory pathways of the early cardiac precursors. Arising from a region adjacent to the developing dorsal lip and Organiser, the precursors move laterally during neurulation due to convergent cell movements. The final movement is ventrally to the lateral mesoderm, the tissue from which the heart is derived

Cardiac differentiation is manifested by expression of markers of cardiac muscle contractility, which confirms its phenotype. Such examples include Myosin Light Chain 2 and Myosin Heavy Chain (MLC2 and MHCα respectively) and Cardiac Troponin (CTnI). *MLC2* expression begins at stage 28 in the presumptive heart region, and at later stages in both atrial and ventricular chambers (Chambers *et al.*, 1994). In association with thin actin filaments, *MHCα* and *MLC2* form cytoskeletal motor proteins that constitute the thick muscle filaments. Through a complex polymerisation process and association with many other constituent proteins they form the cardiac muscle sarcomere, in which contraction is achieved by ATP-dependent movement of the motor proteins (reviewed by Gregorio and Antin, 2000). *CTnI* is a cardiac-specific marker restricted to the myocardium, whose expression begins at stage 28 coinciding with heart tube formation, 24 h prior to beating tissue (Drysdale *et al.*, 1994). Following commitment to the cardiac program and expression of differentiation markers, the linear heart tube then undergoes complex morphogenesis and septation to form the complex chambered structure of the adult heart.

1.2.2.2 Early heart morphogenesis

Heart tube formation marks the beginning of a complex series of structural changes that permits the development of the cardiac arrangement of a particular organism, and is a process that involves a variety of signalling molecules and pathways. Cardiac morphogenesis involves distinct topological events, with gene mutations identifying particular molecules important in each step of the process (reviewed by Fishman and Chien, 1997).

As the heart tube forms and possibly prior to this, it exhibits a distinct A-P arrangement ensuring correct flow of blood and arrangement of the heart chambers. The process is thought to involve distinct expression patterns of a number of important genes, such as the T-box transcription factor *Tbx5* which exhibits a gradient from anterior to posterior (Christoffels *et al.*, 2004). This restriction of particular gene expression to certain regions of the heart occurs for a variety of genes. For example, the basic helix-loop-helix transcription factors *Hand1* and *2* are expressed in the left and right ventricles exclusively, and their double knockout results in lack of looping or ventricle formation. There is redundancy of the involvement of these proteins as knockout of either *Hand1* or *2* alone does not affect looping (Srivastava *et al.*, 1995). Other factors also involved include *MLC1v* and the vitamin A derivative Retinoic Acid (Collop *et al.*, 2006). Once formed, it almost

immediately begins the lifelong pumping contraction movement characteristic of the fully developed heart, initially beginning as erratic peristaltic movements that swiftly adopt a sequential manner (Fishman and Chien, 1997).

The heart tube then begins to undergo a spiral like movement in a process termed *'heart looping morphogenesis'*, a complex process in which the tube moves dorsally and rightward (figure 1.16; Lohr and Yost, 2000). During this phase the ventricular sacs begin to develop, and then expand to give rise to the early chamber regions of the heart that are now aligned in the relative positions they occupy following organogenesis. The result is a laterally asymmetric heart that is characteristic to the vertebrates.

The morphogenetic process is finalised by a thickening of the ventricular walls and development of the atrioventricular valves that permit uni-directional flow of blood. Septation occurs dividing the heart into its respective chambers, with further spiralling of the heart tube. The heart is finally composed of a thick walled ventricle receiving blood from 2 thin walled atria. The developed heart now undergoes a functional maturation and regulation of a uni-directional conduction that arises from careful sequential nerve action potentials, giving rise to the rhythmical contraction between the atria and ventricles (Fishman and Chien, 1997).

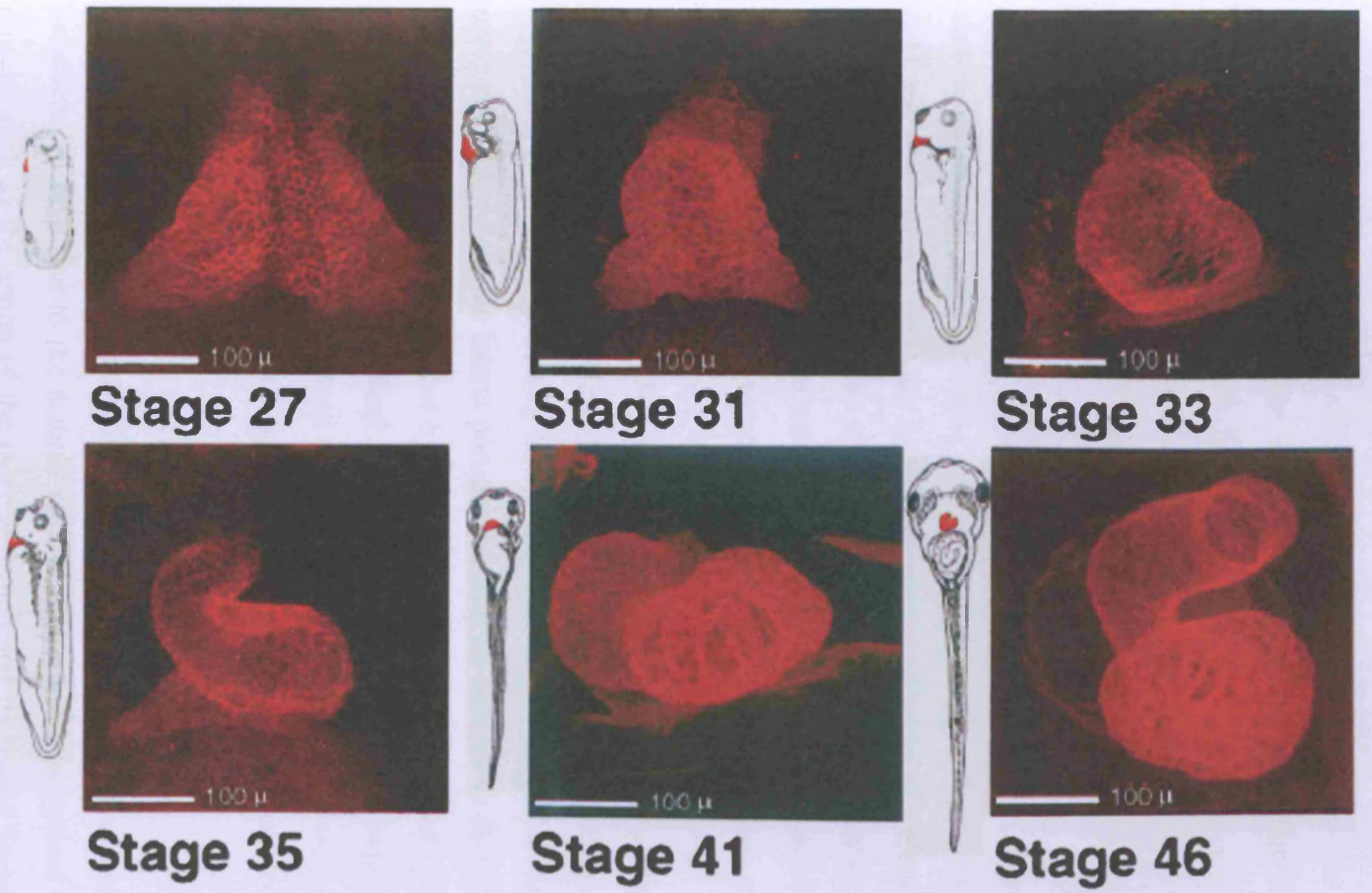


Figure 1.16 – Confocal imaging of early heart morphogenesis

Taken from Kolker *et al.* (2000), once cardiac precursors fuse, a contiguous sheet of cardiac mesoderm is formed. This undergoes morphogenesis to form the primitive heart tube, which undergoes looping and subsequent septation and thickening to form the adult heart

1.2.2.3 Cellular competence and the heart field

Many signalling events often give rise to regions of developmental competence that can respond to an inductive signal. These competent regions often compromise the fated tissue and its surrounding, a so-called morphogenetic field. The heart develops in a specific region termed the 'heart field' (Sater and Jacobson, 1990a). The early heart field at stage 19 is restricted to ventral and lateral portions of anterior regions (figure 1.17a). Eventually however the heart only forms in the most ventral mesoderm. The early heart field comprises a much larger region of mesoderm that can give rise to the heart, which progressively becomes restricted through embryogenesis.

Firstly, Goldstein & Fishman (1998) in zebrafish demonstrated that anterior end of the notochord inhibits posterior expression of *Nkx2.5* in the embryo, limiting the precardiac field and generating a posterior border for heart development. Hence, heart induction is repressed by signals from axial structures. Further work by Tzahor & Lassar in (2001) showed heart induction is prevented by inclusion of either notochord or neural tube with explants of anterior paraxial mesoderm that in their absence show expression of cardiac markers. This could however be reversed by addition of Wnt antagonists and re-introducing BMP signalling. Similarly, in *Xenopus* heart field size is restricted by dorso-anterior structures (Garriock and Drysdale, 2003). It is thought that the entire Nkx2.5-expressing heart field (including lateral and ventral portions) is initially fated to become myogenic. However, the lateral portions are later re-directed to form non-myogenic tissue through inhibition brought about by these axial tissues (Raffin *et al.*, 2000). This re-directs myogenesis downstream of Nkx2.5 preventing contractile gene expression. The evidence for this interpretation comes from the finding that explants of lateral parts of the heart field that do not normally form heart, develop into myocardium once removed from the embryo (Sater and Jacobson, 1990a).

The process of heart field restriction is therefore thought to be a careful patterning of the mesoderm to give rise to the distinct regions of the heart comprised of muscle and non-muscle, such as the action of the receptor Notch and its ligand Serrate, in which Serrate downregulates myocardial expression (Rones *et al.*, 2000). The early cardiac field as marked by *Nkx2.5* expression, is much greater than the region that eventually becomes heart. Recent evidence has suggested the involvement of two mesoderm populations or heart fields (a primary and secondary heart field) which express distinct and overlapping

markers (Tzahor, 2007). The primary heart field comprises the anterior region of the heart field, and forms the left ventricle and atria, whereas the secondary heart field comprises the cardiac outflow tract, right ventricle and atria. Quite how these two distinct populations are integrated into a single organ and the molecular events involved are relatively unknown. It is thought to involve positive signals from the anterior endoderm and lateral mesoderm, and negative signals from axial (BMP antagonists) and neural tissues (canonical Wnts), the sum of which determines the heart border. Its regulation therefore requires combination of similar overlapping signals (figure 1.17; Dunwoodie, 2007).

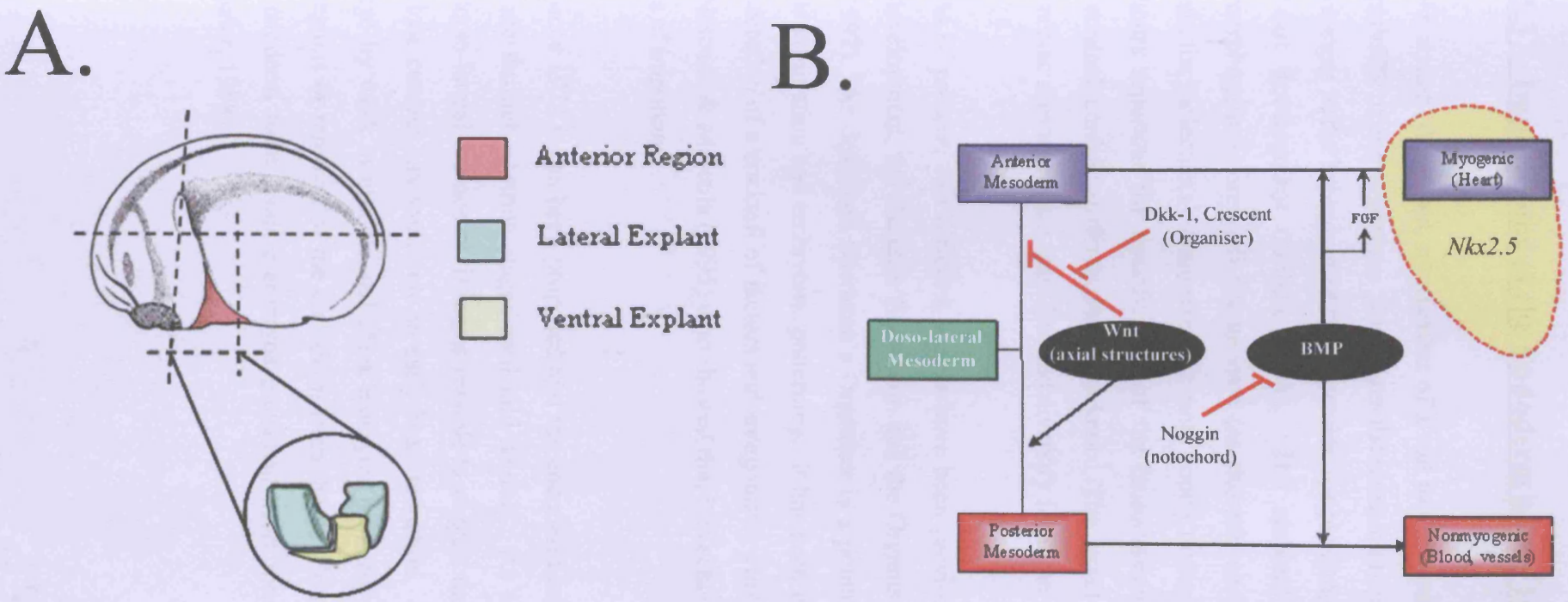


Figure 1.17 – Regulatory network governing the specification of the 'Heart field'

[A] Adapted from Mohun *et al.*, (2003). Schematic of the origins and migratory pathways of early cardiac precursors. **[B]** Adapted from Gilbert (2006). The vertebrate heart derives from dorsal lateral mesoderm. Its competence to enter cardiac morphogenesis is specified by positive and negative regulators, ensuring the only the anterior half develops into heart tissue (demarcated by *Nkx2.5* expression). Axial structures, such as the neural tube, secrete Wnt proteins that inhibit heart formation in the anterior mesoderm.

1.3 Implications of the Endoderm as the Inducer of Cardiac Fate

As already discussed, recruitment of a cell to the cardiac lineage is the first step along the pathway of cardiogenesis. It involves the inducing tissue which secretes a signal that drives changes in the behaviour of the responder, the presumptive cardiac mesoderm in the case of heart development (Gilbert, 2006). The responding tissue, its cellular events and morphogenesis are well documented (as shown); early specification, the signalling tissue and the molecule(s) themselves are more poorly understood. In *Xenopus*, approximately 24 hours separates the specification of the mesodermal germ layer and the expression of terminal cardiac differentiation markers. The exact mechanism by which cells of the cardiac mesoderm are specified and diversify from the mesodermal layer is unknown.

As of present, two different tissues have been previously proposed as regulators of heart development; the Anterior Endoderm and the Organiser (reviewed in Fishman and Chien, 1997). As described Spemann's Organiser is a potent signalling centre important in axis determination and embryonic patterning. It has been implicated in cardiogenesis due to its secretion of a cocktail of factors and antagonists. However, its role has been disproved by Nascone & Mercola (1995) who showed that it is actually the Anterior Endoderm (AE) that is of importance.

Since 1924 it has been proposed that the endoderm and mesoderm interact, and this is the case through gastrula stages until mid neurula. As early heart tissue develops from the dorso-lateral mesoderm, it seems feasible to suggest the underlying endoderm to which it is close contact has some role in early heart induction. This was indicated almost 75 years ago by work in urodele and chick embryos, primarily using explants of prospective heart regions or removal of the endoderm from the developing embryo. Removal of the entire endoderm from urodele embryos prevented heart formation (reviewed by Jacobson and Sater, 1988).

1.3.1 Formation of the Anterior Endoderm

The endoderm is derived from vegetal cells and is committed to its fate during gastrulation. Recently a signalling centre was identified within this tissue in the most anterior region (Jones *et al.*, 1999). Jones and colleagues characterised this tissue's inductive nature, subsequently termed the Anterior Endoderm (AE) by its ability to produce cement glands and impart anterior structure in endoderm explants recombined with pluripotent animal cap cells. Similarly, the mouse anterior visceral endoderm (AVE; homologous to the AE in *Xenopus*) has also been shown to confer anterior identity. Removal of the AVE from mouse embryos severely affected patterning of the rostral endoderm, and was therefore deemed important for anterior patterning (Thomas and Beddington, 1996).

The AE in *Xenopus* at gastrulation was found to be marked by the expression of the homeobox gene *Hex,* a marker of A-P asymmetry and member of the antennapedia/ftz class in *Drosophila* (Newman *et al.*, 1997). *Hex* expression begins at stage 8, but peaks at early gastrula stages in regions confined to the dorsal half in deep endodermal cells fated to become the AE (figure 1.18; Jones et al., 1999). At later stages, Hex is found to be expressed in the liver, gut and thyroid in both the mouse and the frog (Newman *et al.*, 1997; Thomas and Beddington, 1996). The *Hex*-expressing AE itself later in development is destined to give rise to endodermal foregut derivatives, including oesophagus, lungs, stomach, liver, pancreas, and hepatobiliary system (Zorn and Wells, 2009).

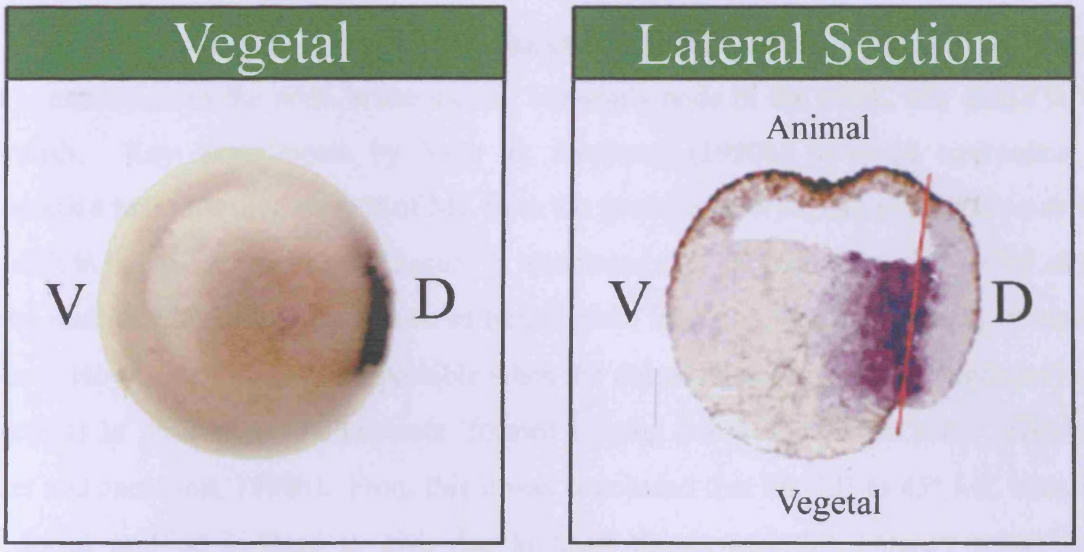


Figure 1.18 – Expression of the homeobox gene *Hex* in *Xenopus*

Taken from Zorn *et al.* (1999). In-situ hybridisation experiments showing expression of the gene *Hex* in stage 10 embryos. The lateral sagittal section depicts the endoderm/mesoderm boundary (red line), and as can be seen Hex is distinctly expressed in the dorsal region of the endoderm, adjacent to the developing dorsal lip.

Zorn *et al.* (1999) found that induction of the AE occurs via combinatorial action of maternal Wnt and endodermal TGF-β proteins. This resulted in formation of a distinct population of *Hex* expressing cells. It was subsequently found that *Hex* contributes to anterior identity, acting as a transcriptional repressor of dorsal mesoderm to maintain the AE (Brickman *et al.*, 2000). Injection of a construct of *Hex* fused to the transcriptional activator domain VP16 resulted in anterior truncations and expansion of dorsal mesodermal structures such as the notochord and somites. Injection of *Hex* in dorsal blastomeres however resulted in loss of somitic tissue and expansion of yolky endodermal like cells, and occurred non-cell autonomously. Consistent with this, it was found that overexpression of *Hex-VP16* resulted in decreased expression of the AE marker *Cerberus* and expansion of the dorsal mesodermal marker *Gsc*. It however, does not inhibit all genes of the Organiser, and *Hex* expression itself is maintained by the BMP antagonists Noggin and Chordin (Jones *et al.*, 1999). Therefore Hex regulates formation of anterior structures by (1) cell autonomously inhibiting *Gsc* and *Chordin* that form axial mesoderm, and (2) activating genes such as Cerberus that promote anterior structure formation (Brickman *et al.*, 2000).

1.3.2 The Anterior Endoderm in Cardiac Specification

As already mentioned the dorsal midline marks the dorsal half of the gastrulating embryo and is analogous to the node in the mouse, Henson's node in the chick, and shield in the zebrafish. Key experiments by Sater & Jacobson (1990b) involved extirpation of prospective heart forming regions of MZ from the gastrula embryo, and scoring their ability to result in the formation of heart tissue. It was found the marginal tissue 60° to 90° of the dorsal midline gave robust formation of heart tissue, in contrast to more lateral or ventral tissues. However, this was only possible when the dorsal most 60° (i.e. the Organiser) was present as in its absence the explants formed beating tissue with much lower efficiency (Sater and Jacobson, 1990b). From this it was concluded that the 30° to 45° MZ lateral to the dorsal midline is fated to give rise to heart tissue, and this potency arises from interactions with the Organiser. This was confirmed by performing the classic Spemann-Mangold transplantation assay, in which the Organiser was transplanted to the ventral half of the embryo causing duplication of the body axes. Sater and Jacobson (1990b) confirmed that upon doing so formation of beating tissue results, and thus the Organiser dorsalises the ventral mesoderm that would not normally give rise to cardiac tissue.

The explants performed by Sater and Jacobson (1990b) were acknowledged to be complex MZ explants containing a degree of mesoderm, deep endoderm, and ectoderm, which included the Organiser tissue. The Organiser transplantation assay resulted in formation of cardiac tissue but only relatively weakly, in comparison to the explants of more dorsal tissue when the Organiser was included. As a result, work by Nascone and Mercola (1995) aimed to dissect the roles of the tissues further, and found an important role for the AE. They found that in explants 75° to the left and right of the midline (to improve survival of the explant), the frequency of formation of heart tissue was much greater when the AE was included, even in the absence of Organiser tissue. On transplantation of the AE into non-cardiogenic Ventral Marginal Zone (VMZ), beating heart-like structures were observed in comparison to non-cardiac control VMZ. In contrast, the Organiser could only induce cardiac tissue in VMZ explants in the presence of AE. It was also found that the main involvement of both Organiser and AE was at the onset of gastrulation, with less obvious effects if extirpated after stage 10.5 (Nascone and Mercola, 1995). This was consistent with previous findings (Sater and Jacobson, 1989), and suggests sustained contact with the

AE is not needed for heart formation.. It was therefore concluded that the dorsalising activity (TGF-β and Wnt antagonists) of the Organiser is necessary to act upon the endomesoderm from which the heart arises, to permit instructive signals from the AE to initiate cardiac fate (Nascone and Mercola, 1995).

Schultheiss and co-workers (1995) also showed a positive role for the inducing nature of the AE in the quail, but in contrast to the findings from *Xenopus* this could occur in the absence of the Organiser. Furthermore, a requirement for cell-cell contact and short range signalling was shown. For the purpose of comparison of cardiac induction assays, the Posterior Primitive Streak (PPS) can be considered as the avian analogue to the VMZ of *Xenopus,* normally fated to give rise to the blood islands and mesenchyme. Cultivation of the PPS with AE was found to result in increased levels of the cardiac markers *Nkx2.5*, *CTnI* and *vMHC*. Unlike other cardiac inductive models such as Activin-mediated cardiogenesis (Logan and Mohun, 1993), upregulation of cardiac markers was found to occur in the absence of skeletal markers. This heart-inducing activity was confined to the anterior endoderm, as recombinants of PPS with posterior endoderm did not result in formation of cardiac tissue (Schultheiss *et al.*, 1995).

Explants of early chick embryo hypoblast (homologous to *Xenopus* pregastrula vegetal region) can promote cardiogenesis in what is normally non-cardiogenic mesoderm (Yatskievych *et al.*, 1997). The cardiogenic potential of different regions of chick blastoderm, consisting of epiblast and hypoblast (which give rise to embryo proper and extraembryonic tissues respectively) was examined. It was found that the more posterior regions of the blastoderm resulted in around 80% formation of cardiac positive tissue, with many cultures exhibiting rhythmical contraction. Expression of *Nkx2.5* occurred in the absence of skeletal muscle tissue. Furthermore, interaction between the hypoblast and epiblast was essential for cardiac formation during early- to mid- gastrula stages. Neither epiblast nor hypoblast formed cardiac tissue in isolation and therefore heart cells arising from posterior epiblast were in response to interaction with the hypoblast (Yatskievych *et al.*, 1997).

Similarly the visceral endoderm of the mouse embryo is required for terminal differentiation of cardiomyocytes. This tissue is homologous to the chick anterior

endoderm that overlies the cardiac progenitors throughout gastrulation and neurulation (Arai *et al.*, 1997). Mesoderm was cultured with various embryonic regions and scored by their ability to form beating tissue. It was found that day 7.5 post coitus mesoderm forms beating tissue, but the more pre-cardiac day 7.25 post coital mesoderm only beat when cultured with visceral embryonic endoderm (Arai *et al.*, 1997). In addition, recent evidence has shown that murine Embryonic Stem Cells (mES cells) could be directed toward cardiac fate by co-culture with the visceral endoderm (Nijmeijer *et al.*, 2009).

1.4 Early Signalling Events of Cardiogenesis

Many developmental processes involve an array of complex signalling cascades that interact in a way that result in a well regulated sequence of events generating tissues and organs, and inevitably forming the organ systems. The development of the heart is a good example of this complex network of regulatory events.

1.4.1 Transcriptional Regulators of Cardiogenesis

One of the difficulties in identifying signalling molecules and pathways involved in cardiac specification is the lack of markers for committed cardiac progenitors. As a result extensive research has been carried out to study the transcriptional regulation of cardiac differentiation. Several important cardiogenic transcription factors have been identified that act in a complex combinatorial manner with requirement for the integration of all these regulatory events (Dunwoodie, 2007). As already discussed however, none of the identified transcription factors acts exclusively in cardiac development (reviewed in Zaffran and Frasch, 2002).

1.4.1.1 The NK homeodomain proteins

The *Nkx* genes are characterised by 4 conserved domains; a short N-terminal TN domain, a homeodomain, an NK domain, and a C-terminal peptide (reviewed by Evans, 1999). At present, 6 *tinman* homologues have been identified, with *Nkx2.5* the most highly conserved. Evidence for their involvement in cardiac development comes from work in *Drosophila.* The *tinman* mutant lacks the fly homologue for *Nkx2.5* with no dorsal vessel (Bodmer, 1993). In vertebrates however, *Nkx2.5* appears to be dispensable for cardiac specification, but it is required for multiple aspects of heart morphogenesis and maturation. *Nkx2.5* is the earliest marker of cardiac progenitors in all species studied (reviewed by Evans, 1999). However, it is not an exclusive marker of those cells as it is also expressed in pharyngeal endoderm and foregut. Its expression begins at stage 14 in a bilobed pattern that coincides with the embryological heart fields, and therefore is a suitable heart field marker (Tonissen *et al.*, 1994) providing a useful indication of cardiac fate. Overexpression of either *Nkx2.5* or *Nkx2.3* causes enlarged hearts in both the fish (Chen and Fishman, 1996) and the frog

(Cleaver *et al.*, 1996), but it is incapable of inducing cardiac differentiation in naïve tissues. Knockdown studies have also revealed that *Nkx2.5* is more likely required to maintain expression of cardiac restricted genes in normal morphogenesis in a diverse number of cardiac developmental pathways, but is not required for heart specification itself (Benson *et al.*, 1999). Acting downstream of the NK family members are other factors implicated in cardiac development. This includes factors such as the MEF2 family of MADS-box transcription factors, HAND transcription factors, and myocardin, a transcriptional cofactor that binds serum response factor (Mohun and Sparrow, 1997).

1.4.1.2 T-box transcription factors

The T-box family of transcription factors is characterised by a conserved T-box binding element and are important evolutionarily conserved transcriptional regulators. Several T-box genes are known to be expressed in the heart, and their importance for cardiac development is exemplified by mutations in these genes manifesting themselves in human genetic disorders. Mutations in *Tbx1* (DiGeorge Syndrome) and *Tbx5* (Holt-Oram Syndrome) result in severe cardiac and vascular malformations suffering early lethality (reviewed by Naiche *et al.*, 2005). In *Xenopus*, both *Tbx20* and *Tbx5* are known to be expressed in the heart (Brown *et al.*, 2003; Horb and Thomsen, 1999 respectively). *Tbx5* expression is exclusive to the heart (and eye) and begins at neurula stages in a pattern complementary to *Nkx2.5*. Using a dominant negative construct for *Tbx5*, blocking its expression resulted in reduced or absent heart formation (Horb and Thomsen, 1999). Similarly, *Tbx20* is expressed from stage 16 in the early heart field and is found throughout the heart in the tadpole (Brown *et al.*, 2003). Knockdown of *Tbx20* expression using antisense morpholino resulted in severe heart defects with reduced cardiac mass, with no chamber formation and lack of characteristic looping. Furthermore, evidence for redundancy is apparent as both *Tbx5* and *20* appear to act in a cooperative manner during heart development (Brown *et al.*, 2005).

1.4.1.3 GATA factors

GATA factors are zinc finger binding proteins involved in regulation of gene expression in mesodermal and endodermal tissue derivatives. GATA factors have a many different roles in embryonic development (Patient and McGhee, 2002). For example, GATA factors are critical for formation of endoderm in the early embryo as part of a conserved network of transcriptional regulators (Woodland and Zorn, 2008). It was found that early activation of

GATAs is the result of Nodal signalling, which are then needed to maintain endodermal expression of these TGFβs. Knockdown of GATA function using MOs resulted in loss of expression of key regulators involved in endoderm development (Afouda *et al.*, 2005). Expressed almost simultaneously with *Nkx2.5* in the precardiac mesoderm (in addition to their endodermal expression), members of this family have been shown to have important roles in cardiac development (Peterkin *et al.*, 2005). Due the combinatorial and overlapping function of these molecules and a degree of redundancy, genetic approaches have been complicated and difficult to interpret (Peterkin *et al.*, 2007; Zaffran and Frasch, 2002). Knockout of GATA4 function in mice results in severe defects in cardiac development exhibiting cardiac bifida (Kuo *et al.*, 1997), but loss of GATA6 results in early embryonic lethality (Koutsourakis *et al.*, 1999). Recently in mice, a cell-autonomous role of GATA4 in heart development has however been shown (Zeisberg *et al.*, 2005) In *Xenopus,* GATA4 and 6 have been shown to be expressed in differentiated heart and gut tissues, and they are detected in cardiac rudiments prior to migration (Jiang *et al.*, 1999; Jiang and Evans, 1996). Further evidence in *Xenopus* (and zebrafish) has shown that knockdown of *GATA6* function using antisense morpholino causes loss of the heart (Peterkin *et al.*, 2003). Initial expression of the early cardiac marker *Nkx2.5* however is unaffected but it was found that cardiac precursors were not maintained. It was therefore concluded that GATA factors are required for maintenance of cardiac precursors (Peterkin *et al.*, 2003). In addition, GATA4 has been shown to be capable of inducing cardiac tissue in the AC, often resulting in the formation of beating tissue (Latinkic *et al.*, 2003). It was found that this could even occur in the absence of endoderm differentiation. It is therefore apparent that GATA factors have essential roles in endoderm formation, and for liver and heart formation. There is however evidence for redundancy. In addition, their involvement seems to be required after specification of cardiac precursors as early transcriptional regulators of the cardiac program (Haworth *et al.*, 2008).

1.4.2 Signals Mediating Cardiac Specification

Although the aforementioned transcriptional regulators are important in heart development, less is known about the signalling molecules that act upstream to control their expression and induce the cardiac program. The lack of molecular markers specifically associated

with cardiac cell fate has meant identification of factors that specify cardiogenesis have not been conclusively resolved. The broad timing window for when specification is thought to occur in addition to identification of the AE as a potential inducer, provided focus for potential signals important in cardiac development. This was further aided by increased understanding regarding the origin of cardiac precursors. As a result, several signalling pathways have been associated with induction of cardiogenesis, many of which were identified by their ability to induce cardiac markers in non-cardiac mesoderm.

1.4.2.1 Bone Morphogenetic Proteins

BMPs have been suggested to be involved in multiple aspects of cardiogenesis with suggested roles in; cardiac specification, heart tube elongation and proliferation, and chamber diversity and formation (reviewed by van Wijk *et al.*, 2007). Early evidence for the involvement of BMP in cardiac specification came from mutant studies in *Drosophila.* The BMP2/4 homologue *Decapentaplegic* (*Dpp*) is required for maintenance of *Nkx2.5* expression, and in its absence the cardiac mesoderm fails to form (Frasch, 1995). Evidence from the chick has shown a necessary requirement for BMP2 in cardiac induction. Schlange and colleagues (2000) investigated the specification of non-cardiogenic central mesoderm, and found that there is an increased expression of the cardiac markers *Nkx2.5*, *GATA* factors, and HAND proteins, following addition of BMP2/4/7. It was also noted however, that administration of the BMP antagonist Noggin prevents myocardial differentiation of the lateral plate mesoderm exhibited by absence of the named cardiac markers, but its effects were less apparent at later stages. It was therefore concluded that there is need for BMP signalling during a short period of cellular competence with a requirement of BMP by cardiac cells until they are determined. This further supports evidence provided in *vivo,* in which it is known that the heart field is negatively regulated by axial structures that express BMP antagonists (Schultheiss *et al.*, 1997; Tzahor and Lassar, 2001), and that the ectoderm overlying the precardiac mesoderm is known to express BMP4, and later BMP2 and 7 (Schultheiss *et al.*, 1997). However, directed differentiation of mES cells towards a cardiac fate was shown not to occur upon treatment with BMP2 or 4 (Yuasa *et al.*, 2005). In contrast, this group found the BMP antagonist *Noggin* to be transiently expressed in heart forming regions of the mouse during gastrulation. They alternatively showed distinct time-windows when reiterative BMP signalling is needed for mesoderm induction and cardiac differentiation, with a period of low BMP signalling important between these events.

There is however, insufficient evidence in vertebrates for an involvement of BMP in cardiogenesis due to the difficulties in separating their roles in different embryonic processes. For this reason the precise requirements for BMP signalling in cardiac development have been difficult to decipher. For example, BMP signalling is involved in D-V axis patterning in the early embryo, and its antagonism causes dorsalisation of the embryo which indirectly affects heart development (Dale and Jones, 1999). It has therefore proved difficult to correlate the role of BMP signalling in cardiac development, and whether it has direct involvement or the results observed are indirect effects attributed to its roles elsewhere. Studies in the mouse with gene knockouts of various BMP ligands and receptors are confounded by embryonic lethality, preventing analysis of involvement in cardiac development (Schneider *et al.*, 2003). *Xenopus* has provided some useful evidence for BMP in cardiogenesis due to selective inhibition of signalling, thus bypassing early patterning defects resulting in lethality. There is however, more evidence to suggest a role in later cardiac development. Selective inhibition of BMP signalling by injection of intracellular BMP antagonists such as the inhibitory *Smad6* resulted in heart defects in late tadpole embryos, but not those of tailbud. It was found that fusion of the early cardiac precursors was inhibited, resulting in cardiac bifida (Walters *et al.*, 2001). It was also discovered that BMP inhibition does not reduce early expression of *Nkx2.5*, but does so at later stages. This was further shown by use of truncated type I and II receptors for BMP signalling that are capable of forming dimers but fail to transduce the signal. Using DNA constructs to prevent interference with gastrulation, embryos in which truncated ALK3 or BMPRII were overexpressed were analysed for reduced cardiac marker expression, asymmetric or delayed expression, or failure of fusion of heart precursors. It was found that these constructs severely affected heart development but early expression of *Nkx2.5* was unaffected (Shi *et al.*, 2000). From these results it appears BMP signalling plays an important role in migration and fusion of precursors, but is not important for early specification events.

Several studies have indicated that BMP is involved in cardiogenesis in a synergistic manner with FGF (Alsan and Schultheiss, 2002; Ladd *et al.*, 1998; Lough *et al.*, 1996; Reiter *et al.*, 2001). Zebrafish mutants for BMP2 (*swirl*) have been shown to exhibit severe defects in myocardial development with reduced *Nkx2.5* expression (Reiter *et al.*, 2001). In the chick precardiac regions are adjacent to BMP2/4/7 expressing cells, and treatment of anterior medial mesendoderm with these compounds does result in formation of cardiac

tissue which is inhibited when BMP signalling is blocked (Schultheiss *et al.*, 1997). However, induction of cardiac fate in more posterior mesoderm with BMP was not possible. It has been shown however that more distal mesodermal regions can be made competent to form cardiac tissue by ectopic expression of FGF2 and 4, together with BMP4 (Barron *et al.*, 2000; Ladd *et al.*, 1998; Lough *et al.*, 1996). This would suggest a close cross-talk between the two signalling pathways to drive cardiac development. It is thus apparent that BMP is required for embryonic patterning and formation of endodermal tissue needed for cardiac specification. The evidence would then suggest a requirement for FGF signalling to establish a heart field competent to respond to BMP signalling, which itself is needed to maintain expression of early cardiac markers and also for fusion/migration and/or differentiation. However, the direct involvement of BMP signalling at the time of specification is unclear.

1.4.2.2 Fibroblast Growth Factors

There is various evidence suggesting an involvement of FGF in cardiogenesis (reviewed by Zaffran and Frasch, 2002). Work in *Drosophila* has shown that an FGF receptor homologue is expressed in the heart of the embryo, and a null mutation for this gene (termed *Heartless*) has severe effects on mesodermal migration. In these mutants, defects in a variety of muscle types were observed, with many cell types failing to be induced, notably there was the complete absence of the dorsal vessel. This was attributed to failure of mesodermal tissue to migrate which therefore prevented key signalling interactions that normally result in induction of these tissues (Beiman *et al.*, 1996). In addition, evidence for involvement of FGF in cardiac development is further exemplified by the fact that multiple FGF ligands (FGF1, 2, 4, 7, 12, 13, and 16) and three of the four FGF receptors (FGFR1, 2, and 4) are expressed in the heart of vertebrates.

In the zebrafish, which share a prototypical heart to vertebrates with many of the early signalling events of cardiogenesis in common, several lines of evidence point toward an involvement of FGF signalling. Mutations in zebrafish *Fgf8* (*acerbellar; ace*) have severe defects in heart development, only forming immature heart tubes (Marques *et al.*, 2008; Reifers *et al.*, 2000). *Ace* mutants have weak *Nkx2.5* and *Gata4* expression at early stages, and are later characterised as having small, dysmorphic hearts with strong defects in ventricular development. Injection of *Fgf8* mRNA was found to rescue the mutant, which could also be phenocopied by treatment of embryos with the FGF inhibitor, SU5402

(Reifers *et al.*, 2000). It was noted that heart size and proportionality was affected in *FGF8* mutants, and Marques and colleagues (2008) further investigated the temporal requirement for FGF in heart development. Timed inhibition of FGF in embryos using SU5402 revealed that during specification of cardiac precursors, blocking FGF inhibited both atria and ventricles, whereas after differentiation only ventricles were affected. Therefore, it was concluded that *fgf8* has roles in regulating heart size and proportionality during specification, and ventricular tissue later (Marques *et al.*, 2008). In morpholino studies, it was found that the more apparent effects on ventricular development resulted from diminished differentiation of cardiomyocytes at the arterial pole rather than loss of cells from that of the ventricule (de Pater *et al.*, 2009).

Similarly, evidence in the chick has already been discussed showing a requirement for the chick homologue for the AE in cardiogenesis where removal of this tissue caused a decrease in cardiac marker expression (Schultheiss *et al.*, 1995; Yatskievych *et al.*, 1997). It has been suggested that *fgf8* contributes to the heart inducing capacity of the AE (Alsan and Schultheiss, 2002). Overexpression of *fgf8* in endoderm-depleted heart-forming regions of early chick rescued the expression of cardiac markers, whereas *BMP2* did not. Furthermore, overexpression of *fgf8* in the embryo expands the cardiac field, but only in regions in which BMP signalling is present, suggesting a synergism between BMP and FGF for cardiac development (Alsan and Schultheiss, 2002). This supports earlier evidence that showed that combined *BMP2* and *FGF4*, but neither alone, can induce cardiogenesis (Ladd *et al.*, 1998; Lough *et al.*, 1996). In addition, it has been suggested that the FGF or BMP proteins have specific inducing capacity in cardiac development and are not compensatory. Induction of cardiac tissue was seen to not be possible if FGF2/4 is substituted with FGF7, and neither could BMP2/4 be replaced with other TGFβs (Barron *et al.*, 2000). The concentration of signalling was also found to be critical, with too high a concentration found to change the fate induced from cardiac tissue. This is consistent with more recent evidence that showed BMP signalling regulates FGF, with low BMP found to stimulate FGF signalling but high levels found to repress it (Alsan and Schultheiss, 2002).

However, a role for FGF signalling in cardiogenesis in *Xenopus* has been deemed to be important during later development. Injection of a dominant negative form of the FGF receptor in the embryo, preventing FGF signalling, was shown to result in severe phenotype, including small heart formation (Amaya *et al.*, 1991). It was also found that

SHP-2, a homologue for *Drosophila corkscrew* which functions downstream of FGF, is needed to maintain cardiac precursors. Inhibition of SHP-2 in prospective heart forming regions downregulates early cardiac marker expression and failure of cardiac differentiation was evident (Langdon *et al.*, 2007). Blocking SHP-2 function using a specific inhibitor caused downregulation of *Nkx2.5*, *GATA4/5/6*, *tbx5/20* and *MHCα* expression. This phenotype was phenocopied by inhibition of FGF signalling. In Noonan syndrome, in which SHP-2 is mutated, patients also exhibit a variety of cardiac abnormalities (Langdon *et al.*, 2007).

1.4.2.3 Wnt Proteins

The involvement of Wnt signalling in cardiac development is a much disputed area of research, with strong evidence for both "procardiogenic" and "anticardiogenic" roles (see Eisenberg and Eisenberg, 2006; Eisenberg and Eisenberg, 2007). Initial evidence for the involvement for Wnt signalling in cardiac development was observed in *Drosophila*. *Wingless* (*Wg*) signalling, the fly equivalent to Wnt signalling in vertebrates, was found to be essential for heart development (Wu *et al.*, 1995). Due to its role in body axis formation and segment polarity, a temperature sensitive construct was used to abolish expression of the gene at different developmental time-points. Upon doing so it was found that cardiogenesis was completely abolished, with the greatest severity observed shortly after gastrulation (around 4 to 4.5 h of development). This was distinct from its other roles in development. In particular, *tinman* expression was severely perturbed in the developing cardiac mesoderm, but deemed normal in other mesodermal tissues in which it is expressed (Wu *et al.*, 1995). In contrast however, a deletion of the intracellular Wnt transducer β-catenin in the AVE and notochord in the mouse, using a cre/lox conditional knockout system, led to formation of multiple hearts. This suggests a negative restriction of heart development by the canonical Wnt pathway occurs normally in *vivo* (Lickert *et al.*, 2002). Similarly, the *liebeskümmer* mutant in zebrafish results in hyperplastic hearts that was found to be attributed to the ATPase complex Reptin (Rottbauer *et al.*, 2002). Reptin normally binds the TCF/LEF/β-catenin complex preventing downstream gene target activation. The mutation renders Reptin active and enhances β-catenin repression, and therefore the hyperproliferative heart is the result of dampened Wnt signalling. There therefore exists conflicting evidence for the requirements of Wnt signalling or its antagonism, between invertebrates and vertebrates.

Further evidence for an instructive role for inhibition of Wnt signalling to promote cardiac development has come from work in other vertebrates. In the chick, work by Marvin *et al.* (Marvin *et al.*, 2001) showed that ectopic expression of the canonical ligand Wnt-3a blocked cardiac differentiation. Furthermore, overexpression of the Wnt antagonists Crescent and Dkk-1 in explants of Posterior Lateral Plate (PLP) resulted in the expression of cardiac markers at the expense of blood, the lineage to which it is normally fated to form. It was concluded that control of a Wnt gradient along the A-P axis establishes heart field competence that intersects with a BMP gradient along the D-V axis to give rise to the heart forming region (Marvin *et al.*, 2001). This evidence is supported in *vivo* by the finding that heart induction in explants of anterior paraxial mesoderm of the chick is prevented by inclusion of either notochord or neural tube which are known to secrete Wnt proteins and BMP antagonists. This block in cardiac induction however could be overcome by ectopic expression of Wnt antagonists (Tzahor and Lassar, 2001).

This was further investigated by Schneider & Mercola (2001), who discovered an increase in the early heart field markers *Nkx2.5* and *Tbx5*, and cardiac contractile proteins *MHCα* and *CTnI* in VMZ explants, upon injection of the Wnt antagonists *Dkk-1, Crescent*, and *GSK3β*. In addition, selective overexpression of only *Wnt3/8* in the cardiac-forming Dorsal Marginal Zone (DMZ) prevented cardiac differentiation. It was proposed that the Organiser secretes the Wnt antagonists *Dkk-1* and *Crescent* to provided an area of low Wnt signalling and stimulate the underlying endoderm to secrete a secondary inducing signal (Schneider and Mercola, 2001). Subsequent work showed that in VMZ explants, Wnt antagonists activate ectopic Hex expression which is then thought to lead to the production of the cardiogenic inductive factor (Foley and Mercola, 2005). It was shown that cardiogenesis initiated in the presence of Wnt antagonism occurred non-cell autonomously, and hence a screen for downstream effectors revealed upregulation in Hex expression. This was further supported by morpholino knockdown of Hex blocked cardiogenesis, and it was concluded that it is the transcriptional repressive function of Hex that is important.

Despite the inhibition of canonical Wnts, it has been shown that promotion of the non-canonical Wnt pathway promotes cardiogenesis in *Xenopus* and ES cells (Pandur *et al.*, 2002). As mentioned, Wnt11 is a non-canonical family member whose expression is pronounced in the DMZ in close proximity to the presumptive pre-cardiac mesoderm. It was found that overexpression of Wnt11 in animal pole and VMZ explants showed

increased expression of the early cardiac markers *Nkx2.5* and *GATA4*, and terminal cardiac differentiation markers *MHCα* and *CTnI*, in a comparable level to induction by Dkk-1 (Schneider and Mercola, 2001). Knock-down of Wnt11 by morpholino oligonucleotides reduced cardiac marker expression in DMZ explants. Furthermore, dominant negative Wnt11 abolished Activin-induced cardiac markers in AC, as Activin normally induces Wnt11 expression. Thus Wnt11 was deemed necessary and sufficient for cardiogenesis, and functions via the JNK pathway to promote heart development (Pandur *et al.*, 2002). However, recently another orthologue for Wnt11 was revealed which was reported to have no role in cardiac specification but was required for cardiac morphogenesis (Garriock *et al.*, 2005). Wnt11-R was found to have greater sequence homology with consistent expression patterns to that of other vertebrates. Furthermore it is expressed in the heart at tadpole stages, but morpholino oligonucleotide knockdown of Wnt11-R however did not effect terminal cardiac markers (Garriock *et al.*, 2005). This is consistent with Wnt11 mutants in zebrafish (*silberblick*), which have no obvious effect on heart development (Heisenberg *et al.*, 1996). Despite significant evidence for both positive and negative roles for Wnt signalling in cardiac development, it remains somewhat controversial. Several lines of evidence suggest a more direct role, whereas it is also believed the involvement of Wnt is indirect, only being involved at the level of mesendodermal induction and patterning (Eisenberg and Eisenberg, 2007).

1.4.2.4 Nodal Signalling

Much work has previously shown that Nodal signalling is key pathway involved in mesoderm and endoderm specification and patterning (Shen, 2007; Tian and Meng, 2006). Any perturbations of Nodal signalling in the embryo have severe effects on normal development.

Nodal was originally identified in the mouse, with mutants for this protein characterised by a failure of mesoderm formation and prenatal lethality (Zhou *et al.*, 1993). Mutation of the nodal related genes, *Cyclops* (*cyc*) and *Squint* (*Sqt*) in zebrafish showed an overlapping role in mesendoderm formation. The *sqt; cyc* double mutant showed defects lacking all dorsal mesodermal derivatives including notochord, blood, heart, and gut, with no expression of the mesodermal markers *Gsc* and *Xbra*. Single mutations in these genes result in a less severe phenotype, indicating some redundancy (Feldman *et al.*, 1998). Furthermore, five of the six nodal-related proteins in *Xenopus* (Xnr1, 2, 4, 5, and 6) have been shown to be

potent mesoderm inducers (Jones *et al.*, 1995; Joseph and Melton, 1997; Takahashi *et al.*, 2000), and a block in nodal signalling using the multi-functional antagonist *Cerberus,* and its truncated forms, prevents mesoderm formation (Agius *et al.*, 2000; Piccolo *et al.*, 1999).

As essential factors for mesoderm formation, Nodals were obvious candidates to be somehow involved in formation of cardiac mesoderm. Several lines of evidence for the involvement of the Nodal pathway in cardiac development have come from studies into its co-receptor, *Cripto*. The EGF-CFC family of cofactors includes the mouse members *Cripto* and *Cryptic, One Eyed Pinhead* (*Oep*) in the fish, and *Cripto-related* proteins (XCR1-3) in the frog. These extracellular proteins are characterised by an N-terminal signal sequence, an epidermal growth factor (EGF)-like domain, and a conserved cysteine-rich domain (CFC), and are known to be essential for Nodal signal transduction. Mutations in these cofactors cause defects in mesoderm formation and axis specification (Dorey and Hill, 2006; Gritsman *et al.*, 1999; Schier and Shen, 2000). *Cripto* via its CFC domain forms a co-receptor complex with the Nodal receptor ALK4 , which enhances binding of the ligand and subsequent activation of Smad2 (Yeo and Whitman, 2001) but is not needed for transduction of Activin signalling (Reissmann *et al.*, 2001). In the mouse, *Cripto* is expressed in the blastocyst and primitive streak, with later expression in the heart. *Cripto-1*$^{-/-}$ knockout ES cells were shown to lack formation of spontaneously beating cardiac myocytes with downregulated *MHCα* and *MLC2* expression. This was however rescued by re-introduction of *Cripto-1* (Xu *et al.*, 1998). Furthermore, generation of *Cripto* null mutant mice resulted in defective cardiac mesoderm lacking expression of all cardiac specific markers (Xu *et al.*, 1999). A more detailed analysis into the timing requirements for *Cripto*-mediated Nodal signalling in mice revealed the timing and duration of Nodal signalling is crucial, with a requirement during specification of cardiac precursors. Furthermore, failure to activate Cripto during an early time-window directs cells toward a default neural state (Parisi *et al.*, 2003).

In the fish, mutation in the *Cripto* homologue *Oep* phenocopied that of *cyc*; *sqt* mutants resulting in cyclopia and no mesendodermal tissue formation. These mutants lack all Nodal signalling and overexpression of wildtype *cyc* or *sqt* failed to rescue the phenotype, emphasising the importance of *Oep* in Nodal signal transduction (Gritsman *et al.*, 1999). In addition, it was observed that these mutants showed severe defects in myocardial development with apparent cardiac bifida and reduced *Nkx2.5* expression (Reiter *et al.*,

2001), and thus *Oep*-mediated Nodal signalling has an essential role in heart formation (Griffin and Kimelman, 2002).

Furthermore, overexpression of Nodal-related proteins in the *Xenopus* AC results in strong induction of mesoderm with some evidence of cardiac tissue formation. *Xnr5*, the very potent mesoderm-inducing family member, resulted in expression of the early cardiac marker *Nkx2.5* (Takahashi *et al.*, 2000). Recently, expression of *Xnr1* and *Cripto* in VMZ explants was reported to induce early (*Nkx2.5* and *Tbx5*) and terminal cardiac differentiation markers (*MHCα* and *CTnI*). *Cerberus* was found to be expressed downstream of this, and its overexpression in VMZ itself induced *Nkx2.5*. This led to the conclusion that Nodal signalling via its coreceptor *Cripto*, activates *Cerberus* expression in the AE to drive cardiac induction (Foley *et al.*, 2007)

1.5 Re-evaluating the Evidence of the Current View of Cardiac Specification

Thorough investigation of the role of the endoderm in heart specification has proved difficult as prior to the predicted time of heart specification the endoderm and mesoderm are not distinct cellular populations and are better described as mesendoderm (or endomesoderm). It has been documented that it is very difficult to separate these tissues in isolation prior to heart formation (Jacobson and Sater, 1988; Nieuwkoop and Faber, 1994). There is strong evidence suggesting an initial inductive role for the AE, but the exact timing and mechanisms are unclear. These early experiments have not fully elucidated the exact time-point when cardiac precursors are specified due to an absence of molecular markers associated with them (as reviewed by Lough and Sugi, 2000; Mohun and Sparrow, 1997). Estimation of the time of specification was based upon a low-resolution retrospective readout of cardiac development (formation of beating tissue). The exact time of specification of cardiac precursors can therefore at present only be regarded as an estimate.

In the amphibian, work of Nascone & Mercola (1995) assayed the conditions required for cardiogenesis and has shown the requirement for the endoderm and Organiser; there was no evidence however to show that AE is sufficient to induce cardiac fate. Much work has also

involved tissue explants that are normally fated to give rise to heart, such as the DMZ, and it is therefore unclear whether the cardiogenesis that resulted was instructive or merely permissive in that the AE was promoting differentiation of already committed cardiac precursors (Schultheiss *et al.*, 1995). It is therefore unclear whether (or to what extent) previous models are reporting true *de novo* cardiac induction, as opposed to modification of pre-existent non-cardiogenic mesoderm (VMZ) or enhancement of already specified tissue (DMZ). Some predict that its involvement is a two-step process; firstly the AE induces cardiogenic competence which with combined influence of the Organiser and BMP signalling, specifies the lateral portion of the heart field to develop (Marvin *et al.*, 2001; Schultheiss *et al.*, 1995; Yatskievych *et al.*, 1997). Following this, the AE secretes a variety of factors such as Activin and FGFs that promote the survival of the cardiogenic competent cells. However, the direct action of the AE has not been confirmed and thus far attempts to recapture the activity in explants of this tissue have been unsuccessful (reviewed in Mohun *et al.*, 2003).

Investigations into early signalling events of cardiac specification have hindered by similar problems. The roles of signalling pathways implicated in specification of cardiac tissue (TGFβ signalling, FGF, Wnt) have relied upon their ability to induce genes of terminal differentiation, as no markers exist to trace early cardiac progenitors. In addition, some of the earlier experiments failed to distinguish an involvement of pathways in cardiac development specifically without further analysis of a role in general mesendodermal patterning. The overall culmination of all these problems is that a direct assay for cardiac specification has not been devised, and as a result an accurate description of early cardiac specification has not been made.

Most of what happens at the very beginning of heart development is unknown. The aim of this project is to help improve our knowledge of this important aspect of embryonic development. Improved knowledge of early heart development may enhance the understanding of congenital heart defects and in addition may improve prospects for regenerative repair of heart failure by providing rational strategies for directed differentiation of stem cells. Specifically, an assay that permits investigation of the early specification events of cardiac fate will be developed. The AE has been suggested to be the inducing tissue of cardiogenesis in the embryo; previous experiments however only investigated its requirement for cardiac induction as assays utilised relied upon

modification of pre-existing non-cardiogenic mesoderm. The sufficiency of the AE will therefore be examined by determining its ability to direct cardiac fate *de novo* in a pluripotent responder. Specification, will therefore be the result of inducing signals from the AE. With such an assay, greater characterisation of cardiac development can be achieved.

CHAPTER 2 – MATERIALS & METHODS

2.0 MATERIALS & METHODS

2.1 Laboratory Equipment & Reagents

Chemicals and reagents used in experiments are described in the following chapter, with suppliers of specialist antibodies and chromagens named. Additional reagents/chemicals such as salts, buffers and equipment were obtained from (in addition to those mentioned below) Bio-Rad (California, USA), Roche Diagnostics (Mannheim, Germany), Fisher Scientific (Loughborough, UK), Invitrogen (Paisley, UK), Ambion (Cambridgeshire, UK), Promega (Madison, USA), Sigma-Aldrich (Dorset, UK), GE Healthcare (Buckinghamshire, UK) and New England BioLabs (Ipswich, UK).

2.1.1 Sterilisation

All equipment and media involved in sensitive applications such as bacterial cultures or those particularly sensitive to nucleases were autoclaved at 121°C for 20 min prior to use. This included all glassware, bacterial media, injection equipment, pipettes, eppendorfs, and tips. Applications particularly sensitive to nucleases, such as RNA preparation for injection, were carried out in areas sterilised with RNase Zap (Ambion).

2.2 Embryological Methods

2.2.1 Obtaining Xenopus Embryos

For experimentation, *Xenopus* embryos were obtained and cultured as previously described (Sive *et al.*, 2000) either by manual extrusion of eggs or by natural mating. Briefly, adult females were injected in the dorsal lymph sac with an appropriate dose of Human Chorionic Gonadotrophin (HcG, Sigma-Aldrich). Depending upon size of the frog this was typically 800 units. For in-*vitro* fertilisation, females were left o/n at 18 °C in a mating tank and eggs collected by manual extrusion into Petri dishes. Male frogs were sacrificed

by Schedule 1 and using forceps, testes surgically removed and stored in Lebovitz's L15 media (Sigma). Small explants of testes were sliced and eggs coated, and left for 5 min for fertilisation to take place indicated by contraction of pigmentation. 10% Normal Amphibian Medium (NAM; 110 mM NaCl, 2 mM KCl, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.1 mM EDTA, 1 mM $NaHCO_3$) was added and embryos left to rotate for 20 min. Embryos were subsequently de-jellied in cysteine (2.2%, pH 8.0; Sigma) and washed thoroughly in 10% NAM. For Natural Mating, males were also stimulated for sexual activity by injection of 300 units of HcG as in the case of the females, and both male and female left in dark container o/n at 18°C to mate. Embryos were collected from the water throughout the subsequent day and de-jellied.

2.2.2 Staging of embryos

Xenopus embryos undergo regular developmental stages with characteristic morphology. Embryos were staged according to the Normal table of *Xenopus laevis* stage development (Nieuwkoop and Faber, 1994).

2.2.3 Micro-injection of Xenopus embryos

Fertilised embryos were kept in 10% NAM prior to injection, and stage development manipulated by temperature control. For injection, embryos were transferred to 4% Ficoll 400 (Sigma) in 75% NAM supplemented with gentamycin sulphate (50 µg/ml; Sigma). Samples were injected using an IM 300 Micro-injector (Narishige Scientific, Japan), with samples loaded into capillary tubing stretched to provide sharp incision, using a Kopf 720 Needle Puller (Kopf Instruments, CA, USA). Needles were back loaded with sample to be injected, and the needle calibrated to inject 10 nl using an eyepiece graticule. Injection samples were typically co-injected with a mix of rhodamine-dextran (20 mg/ml) and dextran-biotin (25 mg/ml) lineage tracers (Invitrogen) (Latinkic *et al.*, 2003; Sive *et al.*, 2000) at a concentration of 10% of injected fluid. Rhodamine-dextran conjugated biotin lineage tracer under DSR light emits red fluorescence. Fluorescence was viewed using a Leica M216 Fluorescence camera (Milton Keynes, UK) with appropriate filter to ensure

correct targeting of injected constructs. Beyond 4 h post-injections, embryos were transferred to 10% NAM due to defective gastrulation arising from high salt concentrations.

2.2.4 Animal Cap Isolation

Embryos were pre-injected at one or two cell stages were cultured until stage 8.5. At stage 8.5, the embryo shows distinct morphology in which it consists of vegetal yolk mass at the base of the embryo and a cavity at the animal pole, termed the blastocoel. Prior to animal cap (AC) isolation, embryos required removal of the vitelline membrane. This is a clear membrane that surrounds the early *Xenopus* embryo and is not shed until neurula stages. Embryos were transferred to 75% NAM supplemented with gentamycin (50 μg/ml), and with the use of one pair of blunt forceps to hold the membrane, another pair of sharp forceps was used to tear away the membrane, with care not to damage the embryo (Guille, 1999).

To remove AC embryos were flipped so that the animal pole faced upwards. Using two incisions, AC were removed with the smallest area possible to ensure a homogenous cell population. Caps were carefully transferred using fine Pasteur pipettes and cultured until the desired developmental stage in 75% NAM supplemented with gentamycine sulphate (50 μg/ml). Culture medium was cleaned regularly. Upon excision, AC readily begin to close. In experiments in which it was desired to keep AC open, they were cultured in Low-calcium Magnesium Ringer's media (LCMR; 43 mM NaCl, 0.85 mM KCl, 0.37 mM $CaCl_2$, 0.19 mM $MgCl_2$, 5 mM HEPES) and Calcium Magnesium-free medium (CMFM; 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 7.5 mM Tris [pH 7.6]) in a ratio of 1:1 (Lamb *et al.*, 1993), delaying constriction.

2.2.5 Media Inhibitor Treatment

For controlled inhibition/activation of signalling pathways, samples were incubated for the specified time in 75% NAM supplemented with appropriate inhibitor (Table 2.1). Inhibitors were dissolved in DMSO (except LiCl dissolved in water) to give a stock and

diluted accordingly to give the final concentration described. Control samples were incubated in DMSO. Verification of inhibitor activity was confirmed using appropriate phenotypic and molecular analysis.

Inhibitor	Source	Mode of Action	Concentration (μM)	Reference
A-83-01	Sigma	ALK4/5/7 inhibitor	75	(Tojo *et al.*, 2005)
SB-431542	Sigma	Blocks ALK4/5/7, ATP kinase	75	(Inman *et al.*, 2002)
SU5402	Calbiochem	RTK inhibitor of FGFR1-4	50	(Mohammadi *et al.*, 1997)
U0126	Sigma	Non-competitive MAPKK inhibitor	35	(Favata *et al.*, 1998)
LiCl	Fisher	GSK3β inhibitor	300	(Klein and Melton, 1996)
BIO	Sigma	ATP binding site inhibitor of GSK3β	*8*	(Meijer *et al.*, 2003)

Table 2.1 – Signalling pathway media inhibitors/activators

A list of commercially available media inhibitors used to abrogate specific signalling pathways, at the concentration described. The inhibitors, via their specific modes of action, block the Nodal (A-83-01, SB-431542) and FGF pathways (SU5402, U0126), and stimulate Wnt/β-catenin (LiCl, BIO).

2.2.6 Dissociation and Reaggregation of Explant tissue

To remove inter-cellular signalling and loosely associated extra-cellular matrix proteins, *Xenopus* explant tissues can be dissociated in low calcium media. Explants (conjugates and AC) were transferred to agarose coated dishes in 95% CMFM in 75% NAM supplemented with gentamycine sulphate (50 μg/ml). Samples were dissociated by gentle pipetting, and the plate gently swirled to collect cells in the centre. Cells were transferred to an eppendorf

tube containing 95% CMFM supplemented with 0.1% Bovine Serum Albumin (BSA; Sigma). Samples were spun at 1,000 rpm for 10 seconds and media removed. 75% NAM was then gently added and samples left to reaggregate for 10 min, by which time they were then transferred to cultivation dishes containing 75% NAM (with gentamycine).

2.3 DNA and RNA Injections

Preparation of material for injectants has been previously described (Sive *et al.*, 2000). Briefly, for DNA, plasmid constructs were injected following purification using Wizard MiniPrep kits (Promega). For RNA injections, linearisation of plasmid constructs and in *vitro* transcription was carried out as described. Concentrations of injectants are described in table 2.2.

2.3.1 Linearisation of template DNA

A list of constructs used in injections is described in Table 3.2. Briefly, DNA (5 μg) was incubated with appropriate restriction enzyme in the associated buffer according to manufacturer specifications. Linearisation was confirmed by agarose gel electrophoresis. Nucleic acids were stained with Ethidium Bromide (5 μl per 100 ml agarose) in TBE buffer (45 mM Tris Base, 45 mM Boric Acid, 1 mM EDTA [pH8.0], 0.1% Acetic Acid) run at 75 V for 30 min. Gels were visualised in UV light (GelDoc-It Imaging System), and indication of molecular weight achieved by incorporation of 1kb marker (Invitrogen). Restriction enzymes were heat inactivated at 65°C for 20 min.

2.3.2 Phenol: chloroform purification of DNA

DNA was diluted with dH_20 (to 100 μl), and Tris-saturated phenol (100 μl; Fisher) and chloroform (50 μl) subsequently added and vortexed, followed by centrifugation at 14,000 rpm for 5 min. The upper aqueous phase was removed and 3 M Sodium Acetate (25 μl) was added, followed by 100% Ethanol (200 μl). Samples were incubated for 15 min at -80°C

followed by centrifugation at 14,000 rpm for 15 min. Following aspiration of supernatant, the precipitate was resuspended in dH_20 (11 µl) and purification confirmed by agarose gel electrophoresis and ethidium bromide staining.

2.3.3 RNA in vitro Transcription

Using linearised templates, the SP6, T7, or T3 promoters of the plasmid was used to drive transcription of the template DNA. Capped transcription products were made to generate stable transcription products, using the 7mG(ppp)G RNA Cap Structure Analogue (NEB). Briefly, DNA (2 µl) was incubated with transcription buffer (400 mM Tris [pH 7.5], 60 mM $MgCl_2$, 20 mM spermidine HCl, 50 mM NaCl; 2µl), 12.5 mM DTT, 1 mM dNTPs, RNAse Inhibitor (RNasin; Promega) and 40 units of SP6 (Ambion), T7 or T3 (Promega), and incubated at 37°C for 2 h. This was followed by addition of RNAse free DNAase (Ambion), and incubated at 37°C for 15 min. RNA was subsequently purified using sephadex G-50 columns (GE Healthcare). Samples were loaded onto columns, spun at 3,000 rpm for 2 min into sterile eppendorf tubes and stored at -80 °C until required. Purity and quantity of RNA was confirmed by running on a 1% agarose gel stained with ethidium bromide, visualised under UV.

Construct	Plasmid	Linearisation	Polymerase	Reference
caALK4 **(1 ng)**	pSP64T	EcoRI	SP6	(Jones *et al.*, 1996)
Cerberus **(1-4 ng)**	pCS2	NotI	SP6	(Bouwmeester *et al.*, 1996)
CerS **(1 ng)**	pCS2	NotI	SP6	(Piccolo *et al.*, 1999)
Dickopf **(1 ng)**	pCS2	NotI	SP6	(Glinka *et al.*, 1998)
FlogNog **(1 ng)**	pCS2	NotI	SP6	Lyle Zimmerman Group, NIMR (Unpublished)
HexVP16 **(500 pg)**	pCS2	NotI	SP6	(Brickman *et al.*, 2000)
LEF-β-catenin–GR **(1 ng)**	pCS2	NotI	SP6	(Domingos *et al.*, 2001; Vleminckx *et al.*, 1999)
Sox17β::EnR **(500 pg)**	Bluescript SK	XhoI	T3	(Hudson *et al.*, 1997)
tBr **(1 ng)**	pSP64T	EcoRI	SP6	(Graff *et al.*, 1994)
XWnt8 **(100 pg)**	pCSKA			(Christian and Moon, 1993)
ΔActRIIB **(1 ng)**	pSP64T	EcoRI	SP6	(Hemmati-Brivanlou and Melton, 1992)
ΔFGFR1 **(1 ng)**	pSP64T	EcoRI	SP6	(Amaya *et al.*, 1991)
ΔTCF3 **(1 ng)**	Bluescript SK	XbaI	T7	(Molenaar *et al.*, 1996)

Table 2.2 – A list of templates used to make mRNA for injection

Plasmid constructs with their restriction sites and polymerases used to generate sense RNA are described (except for Wnt8). Numbers in parentheses denote the amount injected.

2.3.4 Dexamethasone inducible constructs

Glucocorticoid receptor (GR) fusion proteins provide stable constructs which can be injected into the embryo. Fusion to the receptor renders the protein inactive, due to its interaction with Heat Shock Protein 90 (Hsp90). Fused proteins accumulate in the cytosol until its ligand dexamethasone is added, which causes a conformational change that releases Hsp90 activating the protein, and nuclear accumulation (Mattioni *et al.*, 1994). This then result in rapid activation of downstream target gene expression, and is a very useful tool to control gene expression in *Xenopus* (Kolm and Sive, 1995). LEF-β-catenin-GR (Domingos *et al.*, 2001) was injected as mRNA as described above. Embryos were then cultured as normal, but to activate the protein dex (Sigma) was added at a final concentration of 2 μM at the time desired.

2.3.5 Morpholino injections

Antisense morpholino oligonucleotides (MOs) were used to downregulate gene expression (Dash *et al.*, 1987; Heasman, 2002). MOs were obtained from GeneTools (Philomath, USA), and are described in table 2.3. MOs were resuspended in $_dH_20$, and stored at -20°C.

Morpholino	Sequence	Concentration (ng)	Reference
Cerberus	5'-ACT TGC TGT TCC TGC ACT GTG C-3'	10	(Kuroda *et al.*, 2004)
Hex	5'-CCT TAG CTG TAC GTC ATG GTC GTG G-3'	20	(Smithers and Jones, 2002)

Table 2.3 – Antisense morpholino oligonucleotides used in gene knockdown

2.4 Gene Expression Analysis

2.4.1 Whole-Mount in situ Hybridisation

2.4.1.1 Generation of Probes

For Whole-Mount in situ hybridisation (WMISH), antisense riboprobes were generated according to the method of Sive *et al* (2000), transcribed from linearised DNA. Riboprobes were labelled with digoxygenin- or fluorescein- UTP (dig- or flu- respectively; Roche). DNA templates used were linearised at appropriate promoter site for antisense direction, using corresponding polymerases (SP6, T3, or T7; Promega). Templates, restriction site, and RNA polymerase of riboprobes are shown in Table 2.4. Briefly, the following was incubated for 2 h at 37°C:

0.5 µl 1 M DTT
2 µl 10x Dig-/Flu- RNA labelling mix (Roche)
4 µl 5x Transcription buffer (Promega)
2.5 µl Linear DNA (1 µg/ml)
0.5 µl RNaseIn (10 units, Promega)
90 U RNA polymerase (Promega)
Made up to 25 µl with $_dH_20$

Following this, DNase (1 µl; Promega) and RNaseIn (1 µl; Promega) was added and incubated for 15 min at 37°C. Probes were then purified using sephadex G-50 (GE Healthcare) columns, and verified using agarose gel electrophoresis (1%) visualised under UV via ethidium bromide staining.

Riboprobe	Plasmid	Restriction Enzyme	Polymerase	Reference
MLC2	Bluescript SK	EcoRI	T7	(Chambers *et al.*, 1994)
Cardiac Troponin	Bluescript SK	SalI	T7	(Drysdale *et al.*, 1994)
Cardiac Actin	Bluescript SK	SalI	T7	(Mohun *et al.*, 1984)
MLC1v	pSPORT2	SalI	T7	(Smith *et al.*, 2005)

Table 2.4 – Riboprobes used in whole-mount *in situ* analysis

A list of riboprobes used in WMISH, with appropriate restriction site and RNA polymerase described. Probes were labelled either with digoxygenin or fluorescein.

2.4.1.2 WMISH Procedure

Embryos were analysed for gene expression using WMISH, according to the method of Hemmati-Brivanlou *et al.* (1990). Briefly:

Samples were fixed in MEMFA (0.1M MOPS [pH7.4], 2mM EDTA, 1mM $MgSO_4$, 0.1x Formaldehyde) for 2 h, and dehydrated into 100% Ethanol and stored at -20°C. Samples were gradually rehydrated in 5 min washes in ethanol, followed by three 5 min washes in 0.1% TBS-Tween (TTw; 20mM NaCl, 5mM Tris-Cl [pH7.4], 0.1% Tween-20 [Fisher]). Embryos were subsequently permeabilised by washing in proteinase K (10 µg/ml; Roche) in TTw with minimal rotation on a nutating mixer (VWR, Leicestershire, UK). This was followed by two washes in TTw. Samples were re-fixed in MEMFA followed by several 5 min washes in TTw. Prehybridisation was carried out by incubation in the following hybridisation buffer (250 µl) for 10 min at 60°C:

50% Formamide
5 x SSC (SSC; 0.1 M NaCl, 15 mM Sodium Citrate)
5 mM EDTA (pH 8.0)
10% CHAPS (Fisher)
1 x Denhart's solution (0.02% BSA, 0.02% Polyvinylpyrrolidone [PVP, Sigma], 0.02% Ficoll 400
Heparin sulphate (1 μg/ml; Sigma)
Torula RNA Type IX (1 mg/ml; Sigma)

This was followed by 4 h incubation in hybridisation buffer (0.5 ml) at 60°C for 4 h. Fresh hybridisation buffer (0.5 ml) was added containing appropriate dig- or flu- labelled probe (5-10 μg/ml) and incubated o/n at 60°C.

Samples were drained, and incubated in 25% formamide, 2x SSC, 0.1% CHAPS for 10 min at 60°C, followed by two washes in 2x SSC, 0.1% CHAPS for 15 min at 60°C. Samples were finally washed at least twice in 0.2x SSC, 0.1% CHAPS for a minimum of 30 min at 60°C, followed by washing three times for 5 min in MAB-T (0.1 M maleic acid [Sigma], 0.15 M NaCl, 0.1% triton-x). Blocking was carried out for 1 h at RT in MAB-T containing 2% BMB Blocking Reagent (Roche) and 10% Heat Inactivated Sheep Serum (HSS; Sigma). Secondary antibody binding was carried out o/n at 4°C in MAB-T, 2% BMB, 10% HSS. Antibody concentration depended on nature of probe, with α-DIG (Roche) at a concentration of 1:3000, or α-FLU (Roche) at 1:10000.

Samples were washed for five 1 h washes in MAB-T, followed by a 15 min wash in alkaline phosphatase buffer (100 mM Tris [pH9.5], 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20, 2 mM Levamisol [Sigma]). Colour development was then as follows:

- *5-Bromo-4-chloro-3-indolyl phosphate (BCIP, Roche).* This substrate gives an aqua blue colour when used in isolation. Samples were resuspended in AP buffer, and BCIP (3.5 µl/ml) was added and developed o/n at 4°C in the dark on a shaker. Colour reactions were stopped by thorough washing in TTw, and subsequent fixing in MEMFA. Samples were then gradually dehydrated into 100% ethanol.

- *BMPurple (Roche).* Staining is very strong, and as described is dark purple in colour. Samples were transferred to BMPurple solution (0.5 ml) and colour development observed every 30 min. If necessary, this was continued o/n at 4°C. Colour reaction was stopped as in the case of BCIP.

- *Magenta-Phosphate (Sigma).* Colour substrate of choice for overlapping in *situ* analysis (in association with BCIP), it precipitates as a mauve/magenta colour with AP. In combination, Tetrazolium Red (TTZ, Sigma) was also added, which has been shown to enhance colour staining of this substrate and improve its use in overlapping expression (Stern, 1998). Stock solutions of Magenta-Phosphate and TTZ were made in Dimethylformamide (25 mg/ml). As in the case of BCIP, samples were resuspended in AP buffer, and Magenta-Phosphate (9 µl/ml) and TTZ (9 µl/ml) were added and developed o/n at 4°C.

- *Fast Red (Roche).* For development of lineage tracer, samples were washed in MAB-T, and bleached in 0.2x SSC containing 1% H_2O_2 and 5 % formamide under light, followed by thorough washing in MAB-T. Embryos were then incubated o/n in extravadin AP conjugate (1:10000; Sigma) in MAB-T. Samples were washed three times for 5 min in MAB-T, followed by incubation in 0.1 M Tris (pH 8.2) for 15 min. One tablet of Fast Red was dissolved in 0.1 M Tris (pH 8.2; 2 ml) and 500 µl added per vial. Colour was developed over an hour, followed by washing and fixing. Fast Red stained embryos were stored in MAB-T due to loss of colour in organic solvents. As a result, samples processed for histology (described below) were developed for lineage tracer after sectioning.

2.4.1.3 Double in-*situ* staining

For analysis of two different RNA transcripts, double WMISH was performed. This procedure is as described for single-staining with the following alterations. During hybridisation, a fluorescein and digoxygenin probe is added, with the fluorescein generally for the weaker expressed transcript which is developed first. Following initial staining as in section 2.4.1.2, residual alkaline phosphatase activity was removed by gradual dehydration-rehydration into ethanol, and subsequent fixing in MEMFA for 20 min. Samples were washed in MAB-T and blocked for 1 h at RT in MAB-T, 2% BMB, 10% HSS. Appropriate secondary antibody was added o/n at 4°C, and developed as described.

2.4.1.4 Sectioning of Tissue Samples

Following WMISH, samples were sectioned by the method described by Butler *et al.* (2001). Briefly, samples were fixed in MEMFA for 2 h, and gradually dehydrated into 100% Ethanol and stored at -20°C. Samples were warmed to RT, followed by replacement of ethanol with xylene, with subsequent embedding in 60°C paraffin wax, and orientated appropriately. Samples were cooled and sectioned at 10 μM thickness onto glass slides, and remained in wax until subsequent processing. To dewax, samples were incubated in xylene for 2 min, followed by gradual rehydration into MAB-T. Sectioning was kindly carried out by the histology unit, Life Sciences Building, Cardiff School of Biosciences (http://www.cardiff.ac.uk/biosi/facilities/histology/index.html).

2.5.2 RT-PCR

2.5.2.1 RNA Extraction

Total cellular RNA was extracted using the method of Chomczynski & Sachhi (1987). 12-15 conjugates, 20-30 AC, and 5 embryos were used per sample. Briefly, solution D (4 M Guanidinium thiocyanate, 25 mM sodium citrate, 0.1% sarcosyl, 0.1 M β-Mercaptoethanol [pH 7.0]; 0.5 ml) was added to each sample, vortexed and stored at -20°C. For extraction, 2 M sodium acetate (pH 4.0; 50 μl) was added, followed by water saturated phenol (0.5 ml). Chloroform (100 μl) was added and mixed, followed by incubation on ice for 20 min. Samples were centrifuged at 4°C for 20 min at 14,000 rpm. Ice-cold Isopropanol (0.5 ml) was added to the subsequent s/n and incubated at -20°C for a minimum of 2 h. Following

centrifugation at 4°C for 20 min at 14,000 rpm, the s/n was removed and cold 70% Ethanol added and centrifuged as before. Pellets were then air-dried and resuspended in $_dH_20$ (0.5 µl per animal cap) and subsequent RNA stored at -80°C.

2.5.2.2 Reverse Transcription

For Reverse Transcription-Polymerase Chain Reaction (RT-PCR), reverse transcriptase of extracted RNA was carried out using MMLV RT (Invitrogen) according to manufacturer specifications. The volume containing 2 µg RNA was calculated, and random primers (0.5 µg; Promega) added to a volume of 15 µl. To denature, samples were heated at 65°C for 5 min which were then quickly cooled on ice. The following mix was then added per sample, and incubated at 37°C for 2 min:

5 µl	5 x First Strand Buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$)
2.5 µl	DTT (0.1 M)
1.25 µl	RNAse Inhibitor (40 units; Promega)

To each sample, MMLV RT (1.25µl, 200 units) was added and incubated at 25°C for 10 min, followed by 50 min at 37°C. The reaction was heat inactivated at 70°C for 15 min, and subsequent cDNA stored at -20°C.

2.5.2.3 Gel RT-PCR

Analysis of gene expression levels was confirmed using gel RT-PCR. RT-PCR was carried out using the primers described in table 2.5 at a concentration of 0.1 µM. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to span intron-exon boundaries of *Xenopus tropicalis* orthologues to ensure no genomic contamination. Primer sequence and cycling conditions are described.

Plates were set up with duplicates for each cap sample for each set of primers, with the following mix used per well of the PCR reaction. Cycle and reaction conditions were scrutinised such that product amplification was determined to be in the linear range of each specific PCR product (figure 2.1). The following mix was used for each PCR reaction:

5 µl	5x GoTaq Transcription Buffer (Promega)
0.5 µl	dNTPs (10 mM)
2µl	$MgCl_2$ (2 mM)
1µl	Forward Primer
1 µl	Reverse Primer
0.125 µl	GoTaq Polymerase (Promega)

Made up to 25 µl with $_dH_20$

The PCR was carried out using a MiniOpticon PCR Machine (Bio-Rad) with the following cycling conditions

95°C for 3 min

95°C for 30 sec
Tm°C for 30 sec } x cycles
72°C for 30 sec

72°C for 10 min

Primer	Sequence	Accession Number	Tm (°C)	Product Size (bp)	Cycle Number
Cerberus (+) *Cerberus (-)*	5'-GCT TGC AAA ACC TTG CCC TT-3' 5'-CTG ATG GAA CAG AGA T-3'	CR762343	(Zamparini *et al.*, 2006)		
cTnI (+) *cTnI (-)*	5'-GAG CTT CAC GCA AGA ATT GA-3' 5'-GCA TCA ACG TTC TTT CTC CA-3'	BC088784	58	314	34
Endodermin (+) *Endodermin (-)*	5'-CTC GAA GAG CCT CGA GA-3' 5'-ATT CCG GCT TTC CAG GTA GT-3'	L63543	55	398	34
Eomesodermin (+) *Eomesodermin (-)*	5'-GTG CCC AGG TCT TCC TCT GT-3' 5'-TAG CGC CTT TGT TGT TGG TG-3'	NM_001088341	58	345	30
FGF3 (+) *FGF3 (-)*	5'-GTC ATT TGT TTC CAG ACT TC-3' 5'-TAT CTG TAG GTG GTA CTT AG-3'	Z25539	(Kofron *et al.*, 1999)		
FGF4 (+) *FGF4 (-)*	5'-CCG CTT TCT TTC CAG AGA AAC GAC -3' 5'-GTC CGG TAA AAC CTG GAT ATG AA -3'	X62594	54	177	36
FGF8 (+) *FGF8 (-)*	5'-CTG GTG ACC GAC CAA CTA AG-3' 5'-ACC AGC CTT CGT ACT TGA CA-3'	NM_001090435	54	328	36
FGF9 (+) *FGF9 (-)*	5'-TAT TTC GGT GTG CAG GAT GC-3' 5'-CAG CTC CCC TTT CTC GTT CA-3'	BC170125	58	348	34
FGF20 (+) *FGF20 (-)*	5'-TGC TCT TCA ACG ACC CAC TG-3' 5'-GTT CCC GAA AAA TGC ACT CG-3'	NM_001090297	58	315	34
For1 (+) † *For1 (-)*	5'-AGT GGG AAG ATC TGG AGC AG-3' 5'-TGC ACT GAA CTT CAG TGA GC-3'	BC061668	58	615	35
α-globin (+) *α-globin (-)*	5'-CTG GCC ATC CAC TTC CAT AA-3' 5'-TGT TAA CAC CGT CTA ACC TCA GC-3'	X02796	58	145	32
Goosecoid (+) *Goosecoid (-)*	5'- GGA TTT TAT AAC CGG ACT GTG G-3' 5'- TGT AAG GGA GCA TCT GGT GAG -3'	M81481	58	250	34
Hex (+) *Hex (-)*	5'-AGG CCA GTC AGC GAC TAC A-3' 5'-ATT TCC CTG TGG GTT CTC CT-3'	NM_001085590	58	297	32
IFABP (+) *IFABP (-)*	5'-TAC CCT TGC ACA ACC CTT TG-3' 5'-AAT AGA TGG CCC GTC AGG TC-3'	NM_001085877	58	298	32
LFABP (+) *LFABP (-)*	5'-AAG GGT GTC ACC GAG ATT GA-3' 5'-CCT CCA TGT TTA CCA CGG ACT-3'	AF068301	58	154	32
LURP-1 (+) *LURP-1 (-)*	5'-TTT TGG TCG TTT TGG GGA TG-3' 5'-GCA GAA TTT TGG GGT CTT TGC-3'	NM_001088645	58	301	30
Mesp-1 (+) *Mesp-1 (-)*	5'-AAG AAG GCT AGC CGA AAT CC-3' 5'-CCA TCT GAG CCT GAA GCT GT-3'	DQ096961	58	371	36
MHCa (+) † *MHCa (-)*	5'-ACC AAG TAC GAG ACT GAC GC-3' 5'-CTC TGA CTT CAG CTG GTT GA-3'	NM_001091601	58	597	32
MLC1 (+) *MLC1 (-)*	5'-CGG ATC AAA CAG GAC GAT TC-3' 5'-GAA CCC CTG GTA GTG CAG AA-3'	L38596	58	216	30
MLC2 (+) *MLC2 (-)*	5'-TGT ATC GAC CAA AAC CGT GA-3' 5'-CTT CTG GGT CCG TTC CAT TA-3'	Z33999	58	186	30
MSR (+) *MSR (-)*	5'-CTC AGG GAA TGG AGT GGT CA-3' 5'-TGG CAA CAT TGC TCC ACA ATC C-3'	X93045	58	245	32
Myf5 (+) *Myf5 (-)*	5'-ACG AGC ATG TCA GAG CAC CT -3' 5'-ATC TCC ACC TTG GGC AGT CT -3'	NM_001101779	56	214	34
MyoD (+) *MyoD (-)*	5'-AGA GGA ACC CCA CCA TAA CG -3' 5'-TGA GGT GTA TCG CTT CAG GG -3'	NM_001085897	56	202	32
Nkx2.5 (+) *Nkx2.5 (-)*	5'-GAG CTA CAG TTG GGT GTG TGT GGT -3' 5'-GTG AAG CGA CTA GGT ATG TGT TCA-3'	BC056048	58	250	34
N-tubulin (+) *N-tubulin (-)*	5'-GCA TTG ATC CTA CAG GCA GT-3' 5'-TGG GTC AGT TGA AAA CCT TG-3'	X15798	58	424	28
ODC (+) *ODC (-)*	5'-GCC ATT GTG AAG ACT CTC TCC ATT-3' 5'-TTC GGG TGA TTC CTT GCC AC-3'	NM_001086698	58	220	28
Scl (+) *Scl (-)*	5'-GCA ATG TCC CTA AAG ATG ATG G-3' 5'-CTG CAG TCT CAG CTC CTG CT-3'	AF060151	58	236	34
Siamois (+) *Siamois (-)*	5'-AAG GAA CCC CAC CAG GAT AA-3' 5'-CTG GTA CTG GTG GCT GGA GA-3'	Z48606	58	274	32
SmActin (+) *SmActin (-)*	5'-CCA ATT GAA CAC GGC ATC AT-3' 5'-GCA TGA GGG AGA GCA TAC CC-3'	AY986490	58	314	30
Sox17α (+) *Sox17α (-)*	5'-GAT GGT GGT TAC GCC AGC GA-3' 5'-TGC GGG GTC TGT ACT TGT AG-3'	NM_001088162	58	377	34
Tbx5 (+) *Tbx5 (-)*	5'-TCA GAA CCA CAA GAT CAC ACA G-3' 5'-GCT CAG CTG GCT CTT CAC TT-3'	BC170344	58	354	34
Vent2 (+) *Vent2 (-)*	5'-ACC TGC CAT GGA CTC TCT GA -3' 5'-ATG TCA ACA CAT GGC CCA AT-3'	NM_001088138	56	267	32
XAG (+) *XAG (-)*	5'-CTG ACT GTC CGA TCA GAC-3' 5'-GAG TTG CTT CTC TGG CAT-3'	NM_001086198	(Walters *et al.*, 2009)		
Xbra (+) *Xbra (-)*	5'-CTG GGA TGT TGC CAA TGA GT-3' 5'-GAT GAA AGC CTG GAA TGT GC-3'	NM_001090578	58	282	32

Cont...

Xnr2 (+)	5'-TGG GCA ATC GAT GGA CAT TA -3'	NM_001087967	54	321	34
Xnr2 (-)	5'-TGA CAT GTG GCT TGG CTC TC -3'				
Xnr3 (+)	5'-GTT TCC CCA ATT CAT GAT GC-3'	BC169689	58	206	30
Xnr3 (-)	5'-AGC TCA GCC AAC TTC AGC CTC-3'				
Xnr5 (+)	5'-GGG ATG CCC ACT CTT CTT CA-3'	AB038133	54	318	34
Xnr5 (-)	5'-CTC CGC CAG CCT TAA CTC AC-3'				
Xpd1p (+)	5'-ATT TCA ACA AGG CCC TAG AGA CCT-3'	NM_001090179.1	58	202	35
Xpd1p (-)	5'-ATC GAT GTG GCC TGT CCT GTT TC-3'				
Xpo (+)	5'-GCT GAT TAC CAT TCA TGT GCA G -3'	X58487	56	394	34
Xpo (-)	5'-TCA CCT CTT GTT CTC TGA GCC -3'				

† Extension time of 40 sec at 72℃ was used due to product length

Table 2.5 – Primer list of those used in the RTPCR

The sequence of each primer is shown accordingly, and were obtained from Invitrogen

Following the PCR, products were analysed by agarose gel electrophoresis (1%) gel electrophoresis stained with ethidium bromide visualised under UV. Quantification of PCR products was carried out using gel densitometry using ImageJ (http://rsbweb.nih.gov/ij/), and samples normalised relative to Ornithine Decarboxylase (ODC) levels as a loading control. Gel densitometry readings were semi-quantitative, giving an indication of relative expression levels of different samples. Given the limitations of such analysis, fold induction levels should only be treated as an estimate and not accurate levels of gene expression.

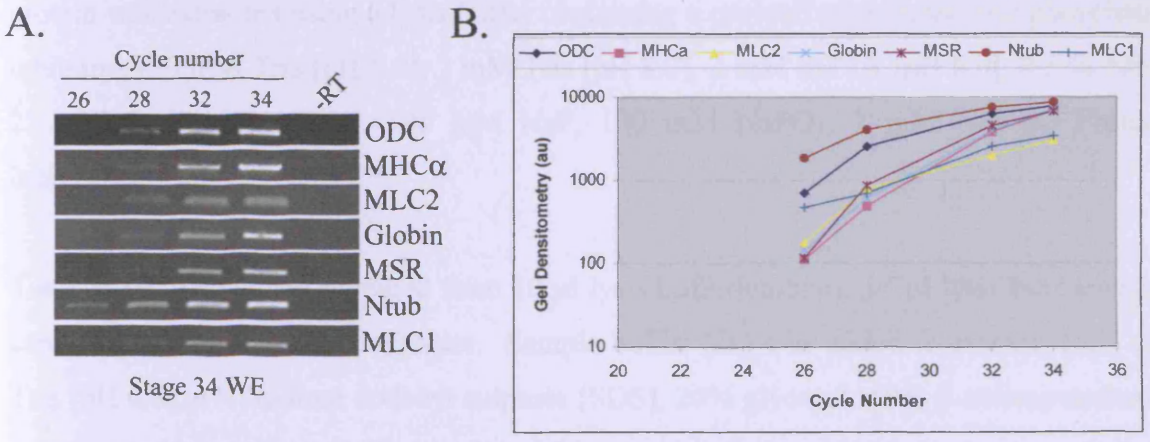


Figure 2.1 - Linearity Control of Gel RT-PCR Products

Cycling conditions for gel RT-PCR were chosen such that the product amplified was in the linear range. **[A]** PCR reactions were set up using some of the frequently used primer pairs for transcripts expressed in stage 34 whole embryo (WE). Samples were taken after 26, 28, 32 and 34 cycles and analysed by agarose gel electrophoresis. **[B]** A plot of gel densitometry readings for each primer pair versus cycle number, confirming linearity of amplified product. Transcripts analysed are; *Myosin Heavy Chain α (MHCα), Myosin Light Chain 2 (MLC2), α-globin, Mesenchyme associated Serpetine Receptor (MSR), N-tubulin (Ntub), Myosin Light Chain 1 (MLC1).*

2.6 Protein Analysis and Western Blotting

2.6.1 Immunohistochemistry

Samples were analysed for skeletal muscle tissue by staining with the monoclonal antibody, 12/101 (Kintner & Brockes, 1984). AC/AE samples, already analysed by WMISH, were rehydrated into PBS-Triton (PBT; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 1% BSA, 0.1% Triton-X). Embryos were then bleached in 30% H_2O_2, followed by washing in PBT and incubation for 1 h at RT. Samples were blocked for 1 h at RT in PBT with 5% HSS (Sigma), and incubated with 12/101 antibody in PBT, 5% HSS o/n at 4°C. Samples were thoroughly washed for five 20 min in PBT, and incubated in PBT, 5% Serum containing Goat α-mouse HRP (1:500), o/n at 4°C. Lastly, embryos were then washed for five 20 min in PBT, and developed with DAB (Sigma).

2.6.2 Western blotting

Western blotting was performed by the method described by Dorey & Hill (2006). Total protein was extracted using a lysis buffer containing a cocktail of protease and phosphatase inhibitors (20 mM Tris [pH 8.0], 2 mM Tris [pH 8.0], 2 mM EGTA [pH 8.0], 0.5 % NP40, 25 mM β-glycerphosphate, 100 mM NaF, 100 mM $NaPO_4$, 1 mM $NaVO_4$, Protease Inhibitor Cocktail [Roche]).

Total cell extracts were prepared from 10 µl lysis buffer/embryo, 1-2µl lysis buffer/animal cap, or 6-8 µl lysis buffer/conjugate. Sample buffer (2x) was added to extracts (125 mM Tris [pH 6.8], 4% Sodium dodecyl sulphate [SDS], 20% glycerol, 10% β-mercapotethanol, 0.01% w/v Xylene Cyanol, Bromophenol Blue) and boiled at 100°C for 5 min. Samples (10 µl) were resolved by SDS polyacrylamide gel electrophoresis in running buffer (0.1% SDS, 25 mM Tris [pH 8.3], 192 mM Glycine) at 200V for 40 min. Gels were made as described in Table 2.6. Proteins were then transferred to Polyvinylidene Fluoride (PVDF) membranes in transfer buffer (25 mM Tris [pH 8.3], 192 mM Glycine, 20% w/v Methanol) at 85V for 90 min.

	10 % Resolving Gel (µl)	Stacking Gel (µl)
40% w/v Acrylamide	2500†	696†
1.5 M Tris-Cl (pH 8.8)	2500	650††
10% SDS	100	100
10% Ammonium Persulphate	50	50
TEMED	20	10
Water	4830	3650

† *37:1 Acrylamide for stacking gel, 29:1 Acrylamide for resolving gel*
†† *0.5M Tris-Cl (pH 6.8)*

Table 2.6 – SDS polyacrylamide gels used in Western Blotting

Membranes were washed for 5 min in TTw, and blocked in SuperBlock (Thermo Scientific, Illiniois) for 1 h at RT. Membranes were then incubated o/n at 4°C in SuperBlock with appropriate dilution of antibody; α-HA (1:1000; Santa Cruz) and α-diphosphorylated-ERK (1:1000; Cell-Signalling Technology). Membranes were then washed three times for 5 min in TTw. For α-diphosphorylated-ERK (α-dpERK), membranes were incubated for 1 h at RT in TTw with α-mouse HRP (1:1000; Santa Cruz) and subsequently washed three times for 5 min in TTw. α-HA was HRP conjugated and required no secondary antibody incubation. Detection was achieved using chemiluminescent detection according to manufacturer (Pierce). As a loading control, membranes were stripped and probed with α-ERK (1:250; Santa-Cruz). To strip membranes, blots were incubated at 37°C for 5 min in Restore Western Stripping Buffer (Thermo Scientific), and subsequently washed three times for 5 min in TTw. Membranes were then blocked and re-probed as before.

CHAPTER 3 – DEFINING A MODEL OF CARDIAC DEVELOPMENT IN *XENOPUS LAEVIS*

3.0 DEFINING A MODEL OF CARDIAC DEVELOPMENT IN *XENOPUS LAEVIS*

3.1 Introduction

As already suggested there are various lines of evidence for the involvement of the AE in cardiac development in a range of organisms (reviewed by Lough and Sugi, 2000), namely the chick (Schultheiss *et al.*, 1995) and the amphibian (Nascone and Mercola, 1995). Efficient cardiogenesis in *Xenopus,* in *vivo* and in explants of the DMZ, requires contact with the AE (Nascone and Mercola, 1995). Similarly, endoderm is required for cardiac specification in chick embryos (Schultheiss *et al.*, 1995; Yatskievych *et al.*, 1997). In previous studies results were unclear regarding the involvement of the AE as the direct inducer of cardiac fate. The myocardial inducing capacity was tested on the ability to induce cardiogenesis in tissue normally fated to give rise to the heart. Therefore it was unclear whether the AE was acting instructively by influencing responding tissue to adopt cardiac fate, or permissively by promoting differentiation of already committed cardiomyocytes (Nascone and Mercola, 1995; Sater and Jacobson, 1990b; Sugi and Lough, 1994). The MZ of the gastrulating embryo consists of cells from all three germ layers, and at gastrulation there is little distinction between these different cell-types (Nascone and Mercola, 1995; Nieuwkoop and Faber, 1994). Even though it was found that the AE does increase the frequency of heart formation, in absence of the Organiser, it was acknowledged that it is possible the AE was acting upon tissue that to some degree had already been dorsalised. This further explains the failure of the AE to specify VMZ tissue (Nascone and Mercola, 1995). It is clear that the AE is involved, but due to low resolution of the models used and difficulty in accurate extirpation of the deep AE, the existing evidence was inconclusive and thus required further investigation.

3.1.1 Chapter Aims

Using the described strategy, it was aimed to address the following questions:

- Can the AE be reproducibly excised from the gastrulating embryo, when cardiac specification is thought to occur, free from contaminating mesoderm?

- Does the aforementioned extirpated AE act as a positive signalling centre and inducer of anterior fate?

- Is the AE in isolation sufficient to induce cardiac specification in pluripotent ectoderm?

3.1.2 Experimental Strategy

The role of the AE as an inductive tissue that is important for anterior development has already been confirmed in *Xenopus* (Bradley *et al.*, 1996; Jones *et al.*, 1999) and in the mouse (Thomas and Beddington, 1996). However, the action of the AE as a direct inducer of cardiogenesis was not addressed.

3.1.2.1 The Animal Cap

A model to investigate whether the AE can respecify competent responding cells toward cardiac fate was devised by utilising pluripotent blastula stage ectoderm (figure 3.1). Previous experiments have shown that in *vivo* the AC normally develops into ectoderm, forming the CNS on the dorsal side due to dorsalising signals from the Organiser (Kuroda *et al.*, 2004). When isolated and cultured in salt media, ACs form atypical epidermis (Guille, 1999). However, these cells show a pluripotent ES cell-like property and can be directed to produce a variety of specific cell types, including heart and vascular tissues, liver, kidney and pronephros, and neural tissue (Asashima *et al.*, 2009; Okabayashi and Asashima, 2003). When using the AC, considerations with regards to their 'default' state must be taken into account. For example, the population of cells in the AC is not uniform with some dorsal-ventral polarity. When AC were dissected into presumptive dorsal and ventral halves and treated with the same concentration of Activin, cells in the prospective dorsal half differentiated into dorsoanterior structures (neural tissue, muscle and notochord). In contrast, ventral cells were directed toward ventro-lateral fates (Sokol and Melton, 1991). This however, can be obliterated by dissociation of the AC in specific media (section 2.2.6). This is thought to eliminate intercellular signalling and remove components of the extracellular matrix, and it leads to autonomous neuralisation (Grunz and Tacke, 1989) presumably by the inhibition of BMP signalling (Wilson and Hemmati-

Brivanlou, 1995). Similarly, the outer pigmented cells that surround the cap are largely unresponsive to inducing signals. For example, a study by Cooke *et al.* (1987) showed that through treatment with Activin protein of freshly open AC explants in the culture media, they elongate due to formation of mesoderm. However, treatment of an AC sandwich in which the explant forms a sphere and only the pigmented layer was exposed, resulted only in atypical epidermis. Thus the pigmented layer of the AC is not responsive to mesoderm induction and does not permit Activin signalling through to the internal layers (Cooke *et al.*, 1987). Furthermore, a study into which the layers of the *Xenopus* AC were separated and treated in isolation revealed that the superficial and sensorial layers respond differently to developmental signals. Treatment of the layers with the same concentration of Activin induced endoderm and mesoderm in the outer layers, whereas only endoderm was induced within the inner layer (Ninomiya et al., 1999). Recently, evidence has suggested that this intrinsic difference between the superficial and sensorial layer may be the result of expression of certain genes conferring a difference in competence (Chalmers *et al.*, 2002). This work was focussed on elucidating the mechanisms by which the AC differentiates into neural tissue, and why the superficial layer has a propensity to form primary neurones and the sensorial layer to form neuroectoderm. They found that general neuronal markers (such as NCAM; Kintner and Melton, 1987) are expressed throughout the AC but differentiation markers (such as Ntub; Oschwald *et al.*, 1991) are only found in the deep layer. The superficial layer was found to be refractory to factors promoting neuronal differentiation, and this was attributed to expression of repressors in the superficial layer. Thus, the AC has differential competence to adopt a particular cell fate (Chalmers *et al.*, 2002).

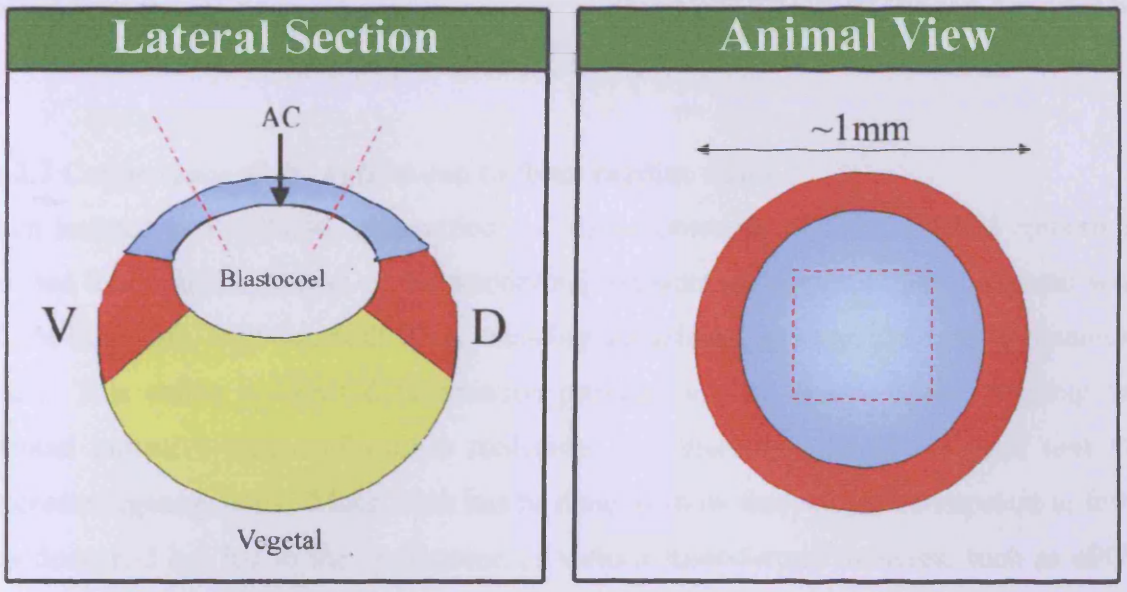


Figure 3.1 – Ectodermal Animal Cap Explants

At stage 8.5 the *Xenopus* embryo the 3 germ layers of the embryo are forming; the ectoderm (blue), the mesoderm (red), the endoderm (yellow). The ectoderm consists of a three cell layered roof at the animal pole called the animal cap, composed of an outer pigmented layer and inner sensorial layer separated from the yolky vegetal pole by the blastocoel. In normal development the AC gives rise to the embryonic ectoderm and CNS. At this stage, it is possible to isolate this region via excisions in this region (depicted by red line), which can subsequently be cultured in salt media

3.1.2.2 Mesoderm Induction in the Animal Cap

Previously the AC has been used to study a variety of inductive events, in particular induction of mesoderm and tissue differentiation. For example it has been used to demonstrate the ability of Activin and FGF to induce a variety of mesodermal/endodermal tissues in a dose-dependent manner (Green *et al.*, 1990). Treatment of isolated AC with varying concentrations of Activin resulted in the formation of different types of mesoderm with a gradual change from ventral mesoderm (mesenchymal) to intermediate mesoderm (muscle-containing) and on to dorsal mesoderm (notochord-containing), with increasing concentration (Green *et al.*, 1990). The TGF-β like factor Activin (named XTC-MIF in the aforementioned study, derived from its isolation from a *Xenopus* cell line) was however deemed 40-times more potent than FGF, with prolonged competence of the AC to respond to its inductive signal up to embryonic stage 11. Finer resolution of this graded response to Activin using dissociated AC cells, showed Activin is able to induce mesodermal cell fates

ranging from the most ventral-posterior to antero-dorsal in a narrow dose range bounded by distinct threshold concentrations (Green *et al.*, 1992).

3.1.2.3 Competence of the animal cap to form cardiac tissue

When isolated and cultured, unspecified AC tissue proceeds to form atypical epidermis. Directed tissue differentiation of the ectodermal explants only occurs upon treatment with soluble inhibitors, injection with RNA encoding an inducer, or conjugation to an inducing tissue. This ability to direct differentiation provides a good means of investigating the potential inductive nature of various molecules and therefore provides a good tool for vertebrate organogenesis. Much work has be done to show that ACs are competent to form mesoderm and has led to the verification of various mesodermal inducers, such as eFGF and Activin, Vg1, and other members of the TGFβ family (reviewed by Smith, 1993). In particular previous work has specifically shown that ACs are competent to be directed toward a cardiac fate (Latinkic *et al.*, 2003; Logan and Mohun, 1993). AC explants treated with high concentrations of Activin protein (80 units/ml) showed expression of the cardiac specific marker *MHCα* in discrete clusters, in addition to other mesodermal derivatives (such as muscle) already shown to be induced (Logan and Mohun, 1993). Consistent with the previous findings that bFGF only induces posterior mesoderm (Green *et al.*, 1992), bFGF was found not to induce cardiac tissue in isolated AC (Logan and Mohun, 1993). More recently, it was shown that cardiac tissue is induced upon injection of the transcription factor GATA4 and also GATA5 and GATA6, as shown by expression of a panel of cardiac specific terminal differentiation markers. Unlike Activin treatment however, GATA-mediated induction of cardiac tissue occurred in a more mesodermal restricted manner with no expression of skeletal muscle tissue (Latinkic *et al.*, 2003). The organogenesis observed in AC explants is not merely restricted to expression of organ-specific markers (in this case cardiac). There is also evidence for cellular diversification and development of more complex tissues, with observation of heart tube formation and fully functional beating tissue (Latinkic *et al.*, 2003; Logan and Mohun, 1993). Work by Asashima and colleagues have taken this further, with formation of beating ectopic hearts observed in mature adult frogs. AC induced with Activin were transplanted into the ventral region of neurula stage embryos and cultured. The result was formation of an ectopic heart that was found to; (1) be incorporated into the host vasculature, and (2) exhibit high order of morphology (Ariizumi *et al.*, 2003). A further advantage of the AC model is that in isolation, caps can be assayed in the absence of cell-cell interactions and restrictive

signalling events that may mask potential signals in the whole embryo. Taken together, the *Xenopus* AC model is therefore a very applicable model system for study into vertebrate organogenesis with reference to cardiogenesis in this case, and the mechanisms that underlie them.

3.1.2.4 The animal cap as a responding tissue

As described, one method to direct fate of AC is to combine them with an inducing tissue to form what are known as conjugates, and thus the cap acts as a pluripotent responder (Gurdon *et al.*, 1989; Jones and Woodland, 1987). The conjugation model devised here was based upon a modification of the original Nieuwkoop mesoderm induction assay (Nieuwkoop, 1969), where it was shown that conjugation of the entire vegetal endoderm to pluripotent AC results in induction and formation of mesodermal tissue that is regionally specified (Dale and Slack, 1987). In the model described here, the initial studies were refined to only the most anterior region (AE) of the gastrulating embryo, as opposed to that of the entire vegetal pole of the late blastula. A more restricted region of the vegetal pole was therefore utilised to form heterochronic conjugates composed of AE taken from stage 10 embryos and stage 8.5 AC.

3.1.3 The Anterior Endoderm/Animal Cap Conjugation Model

To evaluate the role of the endoderm in cardiogenesis, heterochronic cap-endoderm conjugates (AC/AE) were used to analyse the potential of the AE to induce a cardiac fate in the pluripotent explants. To achieve this, endoderm explants were removed from stage 10.25 embryos just as the dorsal lip begins to form, signifying the position of the Organiser, with heart primordia primarily defined as 30-45° to the left and right of this dorsal midline (Sater and Jacobson, 1990b).

To repeat the work of Nascone and Mercola (1995), 150° excisions around the dorsal midline were performed, with subsequent removal of the endoderm from the mesodermal layers. Despite the difficulties in distinguishing between these layers, morphological differences do exist, with endodermal cells appearing larger and yolkier when compared to

those of the marginal zone. A schematic depicting the removal of the endoderm from stage 10.25 embryos is shown in figure 3.2.

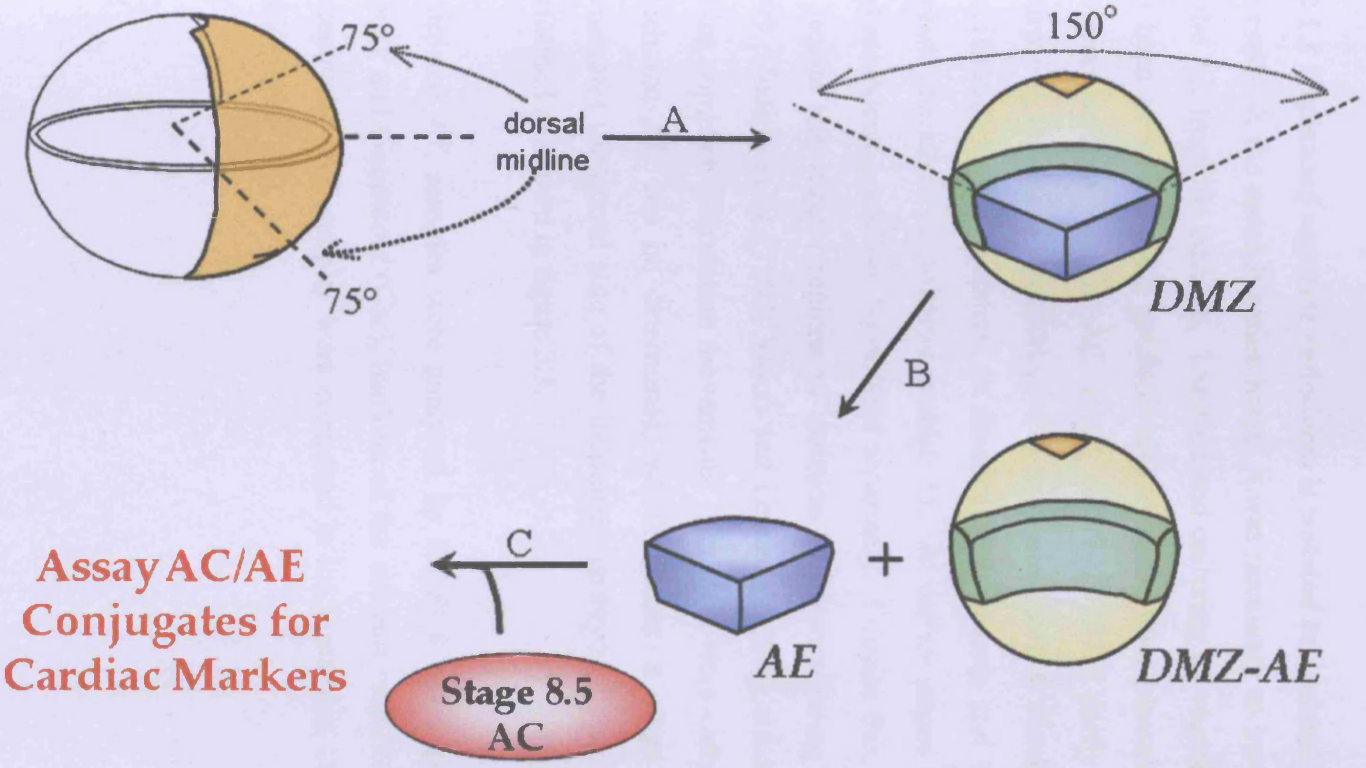


Figure 3.2 – Experimental design for the excision of the AE

At stage 10.25, a 150° excision was made around the dorsal lip, indicated by the pigmentation of the developing blastopore. The ectoderm of the cap was carefully peeled back and explants left for 10 min for distinct morphological differences between mesoderm and endoderm to occur. These larger endoderm cells were subsequently removed using a fine hair loop, with the constriction aiding its separation from the mesoderm. From sibling embryos, delayed so that they remain at stage 8.5 to coincide with stage 10.25 of the AE samples, Animal Caps (AC) were removed. AE was then conjugated to three AC per conjugate and cultured for desired time.

3.2 Results and Discussion

3.2.1 The Role of the AE as an Inducer

3.2.1.1 Explanted anterior endoderm is isolated in absence of mesoderm

To establish the recombination assay, it was necessary to investigate the accurate excision of the AE from the embryo. Excision and culturing of vegetal poles or AE previously has not been as widely (and productively) used as other blastula or gastrula explants of the *Xenopus* embryo, such as AC, DMZ, or VMZ. This partly is attributed to difficulty in extirpating the tissue in isolation. For example, Horb & Slack (2001) investigated regional specification of the endoderm in absence of mesoderm and found it was only possible to explant endoderm in isolation at stage 15. At earlier stages it was deemed the endoderm and mesoderm could not be isolated separately. Despite this, previous work has been able to isolate the vegetal regions in isolation of contaminating mesoderm (Clements et al., 1999; Hudson et al., 1997; Yasuo and Lemaire, 1999), although this was not at the time during complex gastrulation movements. Embryos were cultured until the earliest point at which the AE can be determined, which occurs at stage 10.25 when the dorsal lip demarcates the dorsal side of the involuting embryo (Slack, 1991a). The excisions were performed as shown in figure 3.3.

Extirpated AE samples were analysed by RT-PCR for expression of *XHex, Brachyury* (*Xbra*), and *Goosecoid* (*Gsc*), markers of the anterior endoderm, mesoderm and Organiser respectively. AE samples were compared to the remaining DMZ and the non-cardiogenic VMZ (figure 3.4).

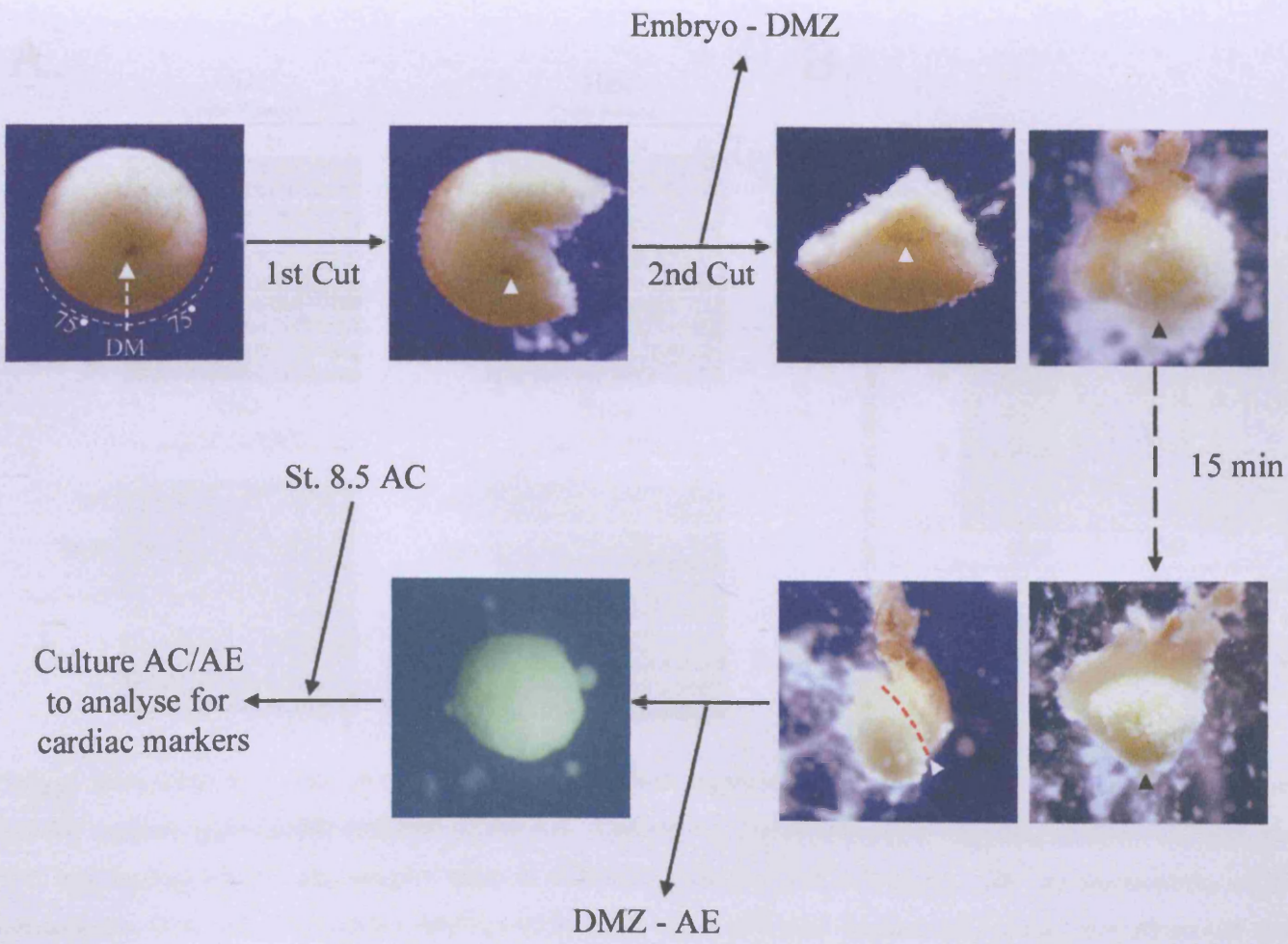


Figure 3.3 – Experimental extirpation of the AE

The heart forming region of the Anterior Endoderm (AE) is deemed to be located within the region 75° to the left and right of the dorsal midline (DM) of a stage 10.25 embryo, marked by the dorsal lip (white arrowhead). Following excision of this region yields the dorsal marginal zone (DMZ). This was allowed to heal for 15 minutes to constrict the cells of the AE, from which they were then separated from the smaller mesodermal cell layers (red dotted line). The resulting excised AE was then conjugated to stage 8.5 animal caps (AC) to form conjugates (AC/AE)

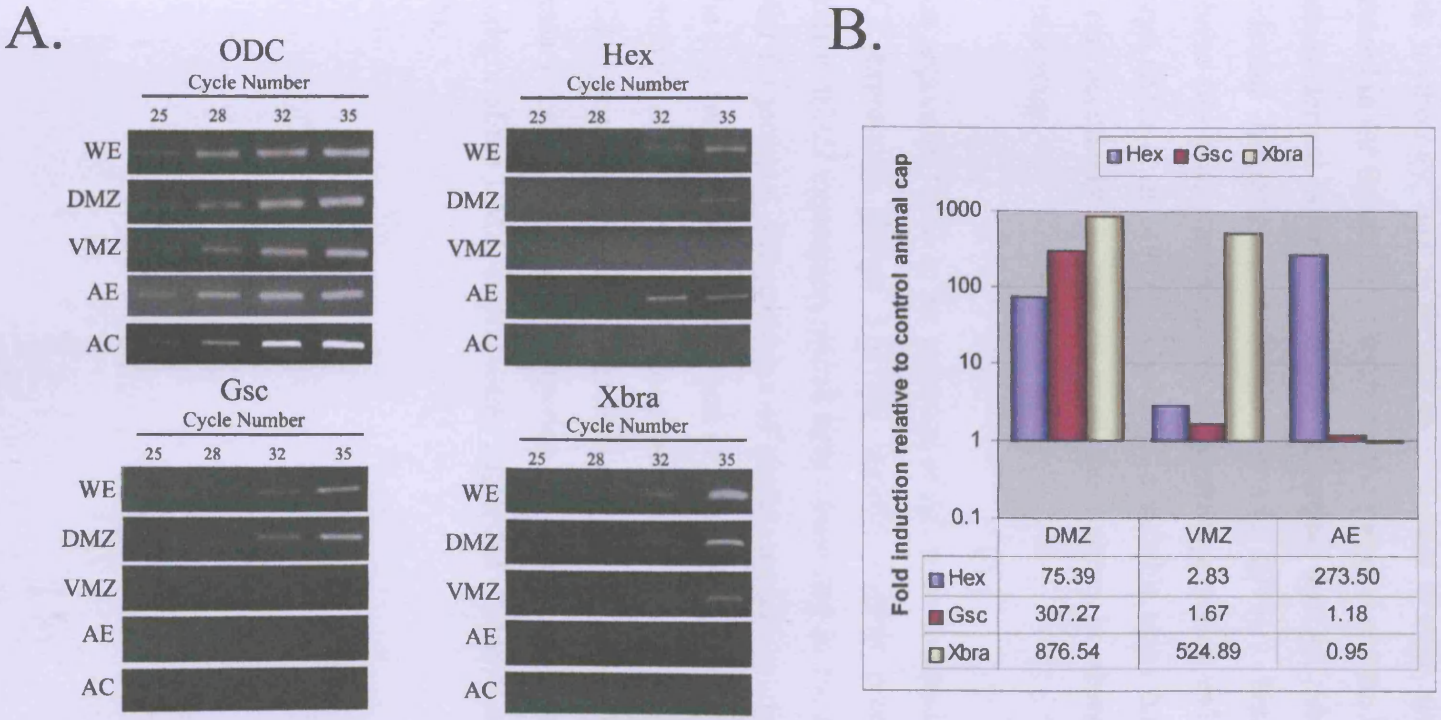


Figure 3.4 – The AE does not express mesodermal tissue, as marked by absence of Xbra expression **[A]** Semi-quantitative gel RT-PCR analysis of stage 10.25 of Dorsal and Ventral Marginal Zone explants (DMZ and VMZ respectively), Anterior Endoderm (AE) and Animal Caps (AC) for expression of markers of mesodermal (*Xbra*), endodermal (*XHex*) and Organiser (*Gsc*) tissue. Whole Embryo (WE) explants were taken as a positive control. The AE explant expresses *XHex*, a marker of AE at stage 10.25, but not mesodermal and Organiser markers, showing accurate reproducible isolation of the AE. Conversely, mesodermal and Organiser markers are both abundantly expressed in the adjacent DMZ. ODC was loading control, and samples taken at different cycles to confirm linearity. **[B]** Gel densitometry of PCR analysis using ImageJ. Samples were normalised to ODC and densitometry readings taken when amplicons were deemed to be in the linear phase (28 cycles for ODC, and 32 cycles for *Gsc*, *Xbra*, and *Hex*).

As shown in figure 3.4a, the domain of the AE is positively marked by *XHex* expression, but lacks both mesoderm and Organiser markers, *Xbra* and Gsc respectively, indicating its accurate excision from the WE. Conversely, the VMZ exhibits some expression of the pan-mesodermal marker *Xbra*, which is abundantly expressed in the DMZ region as is the case for Organiser, marked by *Gsc.* The AE was also shown to express the protein *Cerberus*, a member of the DAN family of secreted proteins (figure 4.4; Bouwmeester et al., 1996). Identified in a screen for dorsal-specific cDNAs, it is named after the two-headed dog from Greek mythology, due to its ability to induce ectopic secondary head structures. It is expressed in the Spemann's Organiser, the secreted protein is expressed at stage 10 in deep endomesodermal layers, which upon injection into AC results in induction of neural and endodermal markers (Bouwmeester *et al.*, 1996). Similarly, the mouse homologue *Cerberus-like* is also expressed in the anterior primitive endoderm of the developing mouse embryo (Belo *et al.*, 1997). Therefore, a suitable assay has been devised from which the AE can accurately be excised from the embryo in absence of mesoderm, at the early gastrula stage.

When regions of DMZ in the presence of AE were cultured until stage 34 and analysed for *MLC2* expression (figure 3.5) the samples exhibit prominent anterior structure with abundant *MLC2* expression, which is not exhibited in the absence of the AE. *MLC2* is a marker for terminal differentiation of cardiac muscle, whose expression begins at stage 28 in the presumptive heart region, and at later stages in both atrial and ventricular chambers (Chambers *et al.*, 1994). AE alone was also cultured until late stage 34, and showed no *MLC2* expression. Evidence therefore suggests that the AE is acting as an inducer and is necessary for the formation of anterior-like structures (including the heart) in the adjacent mesoderm of the DMZ, as previously suggested (Jones *et al.*, 1995; Nascone and Mercola, 1995).

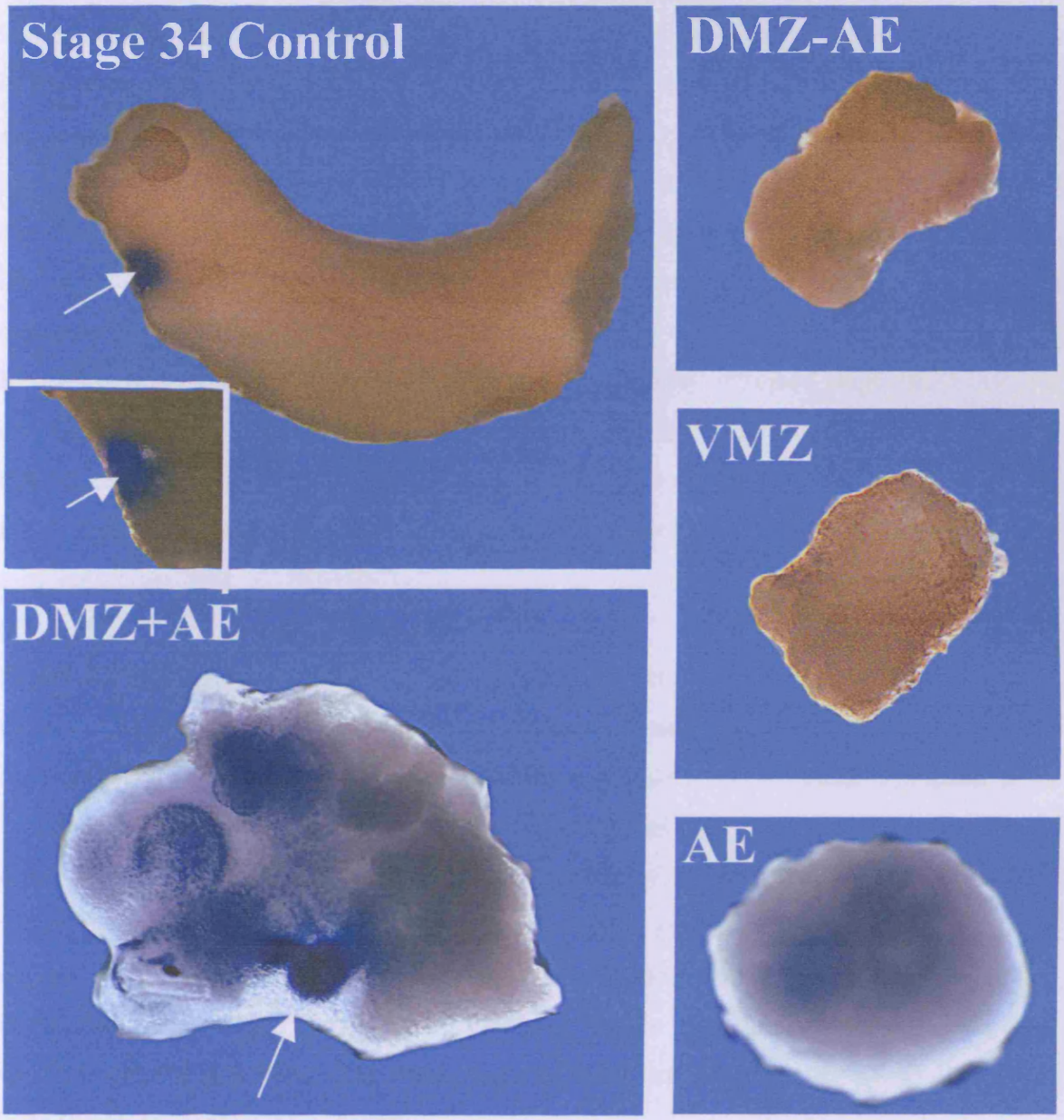


Figure 3.5 – The Anterior Endoderm itself does not give rise to cardiac tissue

At stage 34 when cultured in isolation, the AE does not express *MLC2* (arrow), a marker of the myocardium. When left in DMZ explants however, it is capable of inducing anterior structures including the heart (c.f. DMZ+/- E), verifying its requirement for heart induction.

3.2.1.2 The AE is a positive Inducer of Anterior Fate

The inductive properties of the AE have been confirmed in the amphibian by its ability to induce cement glands in animal caps (Jones *et al.*, 1999), an ability which was reproduced in this study as described above. Also, work by Schneider and Mercola (1999) showed the AE has distinct head and heart inducing capabilities. They suggest that the deep endoderm, marked by *Cerberus* expression is required for heart inducing activity. This is opposed to the suggested heart inducing activity of the *XHex* expressing dorsal anterior endoderm that lies in close contact with the mesoderm (Schneider and Mercola, 1999). When DMZ explants were cultured and *Cerberus* expressing cells removed, the incidence of heart formation was reduced but head formation was unaffected. Excised stage 10.25 AE was conjugated to stage 8.5 AC and cultured until stage 24, when stage control embryos showed distinct cement gland formation in the anterior portion of the embryo. It was determined if the endoderm had the potential to induce such structures, in both conjugates and control samples (figure 3.6).

Figure 3.6 confirms the AE as a signalling centre, depicted by its ability to induce cement glands. VMZ explants show no anterior structure or cement gland formation, as is also the case in DMZ explants from which the endoderm was excised (DMZ-AE). Inclusion of the endoderm however, results in anteriorisation of the explant with what appears as the development of a head-like structure and formation of the cement gland (DMZ). This would suggest that the endoderm has induced anterior structures in the responding DMZ explant. This was also recapitulated by the endoderm induction of cement gland formation in the pluripotent animal cap (AC/AE), verifying its role as an inductive tissue. This also confirms the AC as a responding tissue, forming axial structures that are normally induced in MZ explants containing endoderm.

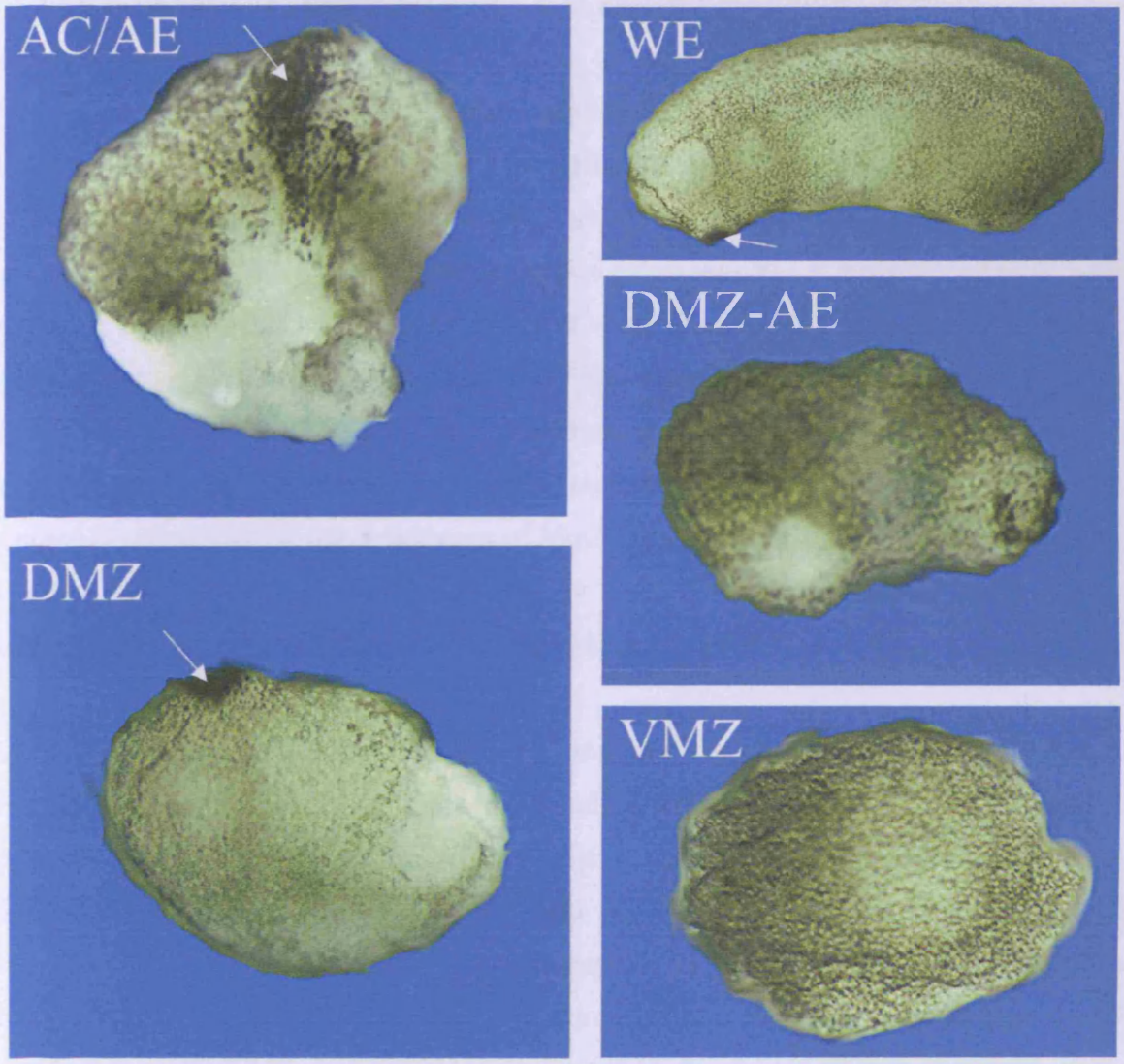


Figure 3.6 – The AE has the capacity to induce anterior fate

The cement gland (arrow) marks the most anterior portion of the whole embryo (WE), at stage 24. Anterior endoderm-animal cap conjugates (AC/AE) or dorsal marginal zone explants (DMZ) similarly possess cement glands. In absence of AE however, neither the dorsal marginal zone (DMZ-AE) nor the Ventral marginal zone (VMZ) exhibit such structures. Thus the AE has a positive role of inducing anterior fate in the embryo.

3.2.2 The Anterior Endoderm in Cardiogenesis

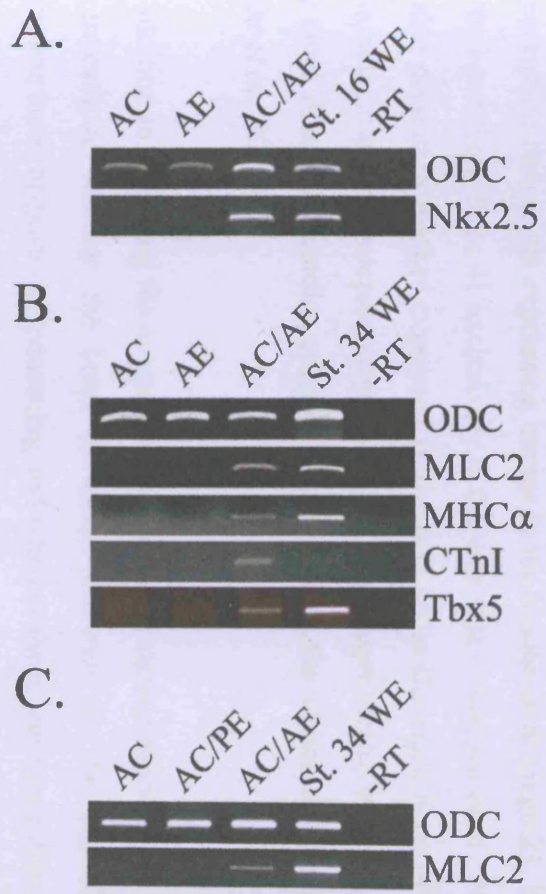
3.2.2.1 Conjugation of ectodermal explants to anterior endoderm results in expression of cardiac markers

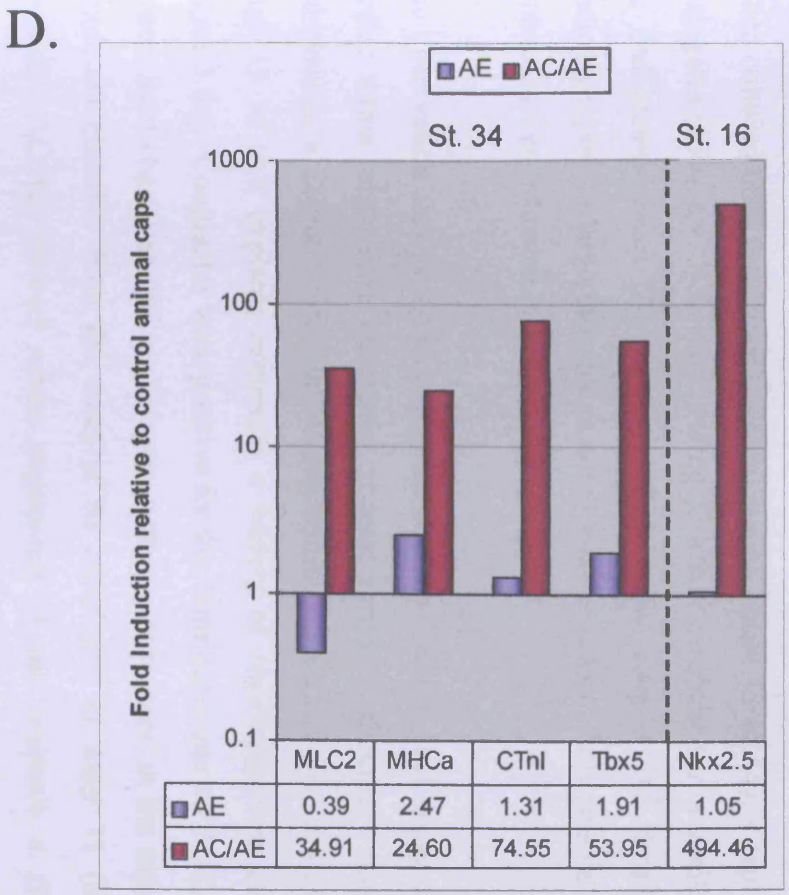
To evaluate the ability of the AE to induce cardiac fate, AC were conjugated to the AE as described (in a 3:1 ratio), and analysed for a plethora of known cardiac markers at different developmental stages. *Nkx2.5* is known to be transcribed during early stages of cardiogenesis and although it is known not to be cardiac-specific, it is often used as an early marker of cardiac fate due to the current lack of early cardiac-specific markers (figure 3.7). It was found that in stage 16 AC/AE, *Nkx2.5* was strongly expressed (figure 3.7a). The *tinman* homologue *Nkx2.5* is known to be involved in various aspects of cardiogenesis, and its expression would suggest a useful earlier marker of cardiac fate in this model. This appears to reflect closely the hypotheses of *Nkx2.5* involvement in *vivo*, unlike in the case of other cardiac models such as induction by *GATA4*, in which cardiac tissue can be induced in absence of *Nkx2.5* expression (Latinkic *et al.*, 2003).

At tadpole stages, AC/AE conjugates were analysed for the expression of a panel of terminal cardiac differentiation markers including *MLC2*, *CTnI*, and *MHCα*. *MHCα* expression begins from the onset of differentiation and is exclusive to the heart anlagen (Logan and Mohun, 1993). It is shown that AC/AE exhibit robust expression of all myocardial markers (figure 3.7b), and also expression of the cardiogenic transcription factor *Tbx5*. Thus, expression of these terminal cardiac differentiation markers would signify that cardiogenesis has not only been specified, but in a sufficient manner to allow expression of differentiation markers. Furthermore, this cardiac inducing capacity was restricted to regions of anterior endoderm, as conjugates of posterior endoderm (AC/PE) did not express cardiac differentiation markers (figure 3.7c).

Figure 3.7 – Animal Cap/Anterior Endoderm conjugates show robust expression of early and late cardiac markers

Animal cap/anterior endoderm (AC/AE) were conjugated as described, and cultured until control siblings reached stage 16 for RT-PCR analysis of *Nkx2.5* expression and stage 34 for analysis of other markers. **[A]** Stage 16 AC/AE strong expression of *Nkx2.5* comparable to the whole embryo, which is not expressed in AC or AE alone. **[B]** Cardiomyocyte specific markers (*MLC2, MHCα, CTnI*), as well as the cardiogenic transcription factor *Tbx5*, are robustly expressed in conjugates at Stage 34. **[C]** Capacity of endoderm to induce cardiac tissue is restricted to the AE as conjugates of posterior endoderm (AC/PE) do not express *MLC2*. **[D]** ImageJ gel quantification of RT-PCR analysis. Samples were normalised against the loading control ODC





	MLC2	MHCa	CTnI	Tbx5	Nkx2.5
AE	0.39	2.47	1.31	1.91	1.05
AC/AE	34.91	24.60	74.55	53.95	494.46

3.2.2.2 AC/AE conjugates express regionally restricted atrial and ventricular markers

When cultured until late developmental stages (stage 43 to 46), a reproducible number of conjugates (55%, n=20) formed beating foci that contracted in a similar manner to that of the endogenous heart (Movie 1). This would suggest the terminally differentiated cardiomyocytes undergo physiological maturation to form fully functional beating cells that contract in a coordinated manner.

The observation that AC/AE conjugates develop beating foci suggested that the induced cardiac tissue might have undergone at least simple rudimentary functional organisation and perhaps a degree of cellular diversification. Accordingly, it was established that at stage 43 AC/AE explants expressed a variety of regionally restricted cardiac markers (figure 3.8a). Conjugates were positive for the ventricular marker *MLC1v*, one of the three myosin light chain variants in *Xenopus*, which is expressed in the myocardial cells of the ventricular chamber from the onset of its expression at stage 31 (Smith *et al.*, 2005). Secondly, AC/AE showed robust expression of the *Iroquois 4 (Irx4)* homeodomain transcription factor. Its expression begins in the hindbrain at stage 21, but is also expressed in the heart from stage 41 exclusively in the ventricle (Garriock *et al.*, 2001). Lastly, *Tbx18* is also present, a marker expressed from stage 41 in the right horn of a bilaterally paired structure called the proepicardium, which through asymmetric morphogenesis gives rise to the outer skin of the cardiac muscle (epicardium) in the advanced stages of heart formation (Jahr *et al.*, 2008).

In addition to analysing the expression of the above named markers, its was also of interest to investigate whether the heart formation observed in the AC/AE model exhibited complete anterior-posterior patterning and whether there was any evidence of atrial specific markers in addition to those of ventricles. Opposed to ventricular markers, markers of the atria are first expressed pan-myocardially and subsequently become restricted to the atria through development, with apparent 'ballooning' of the atria from the heart tube (Lyons *et al.*, 1990). One such marker is *Atrial Natriuretic Factor* (*ANF*), a 28 amino acid circulating peptide whose expression is thought to be regulated by *Nkx2.5*. *ANF* expression begins at stage 32 in all the myocardium, until its expression sharply begins to become restricted to the atria at stage 45. By stage 47, *ANF* expression is exclusive to the atria (Small and Krieg, 2000). Numerous attempts to culture AC/AE until such late developmental stages for analysis of this marker were unsuccessful. As an alternative technique, double in-*situ*

analysis on stage 45 AC/AE was performed. By utilising an appropriate combination of colour substrate, it is possible to distinguish between domains of overlapping expression. Analysis for *MLC2* in the WE revealed pan-myocardial expression, with subsequent chamber restricted *MLC1v* expression overlapping a region of the *MLC2* domain. A resulting region of *MLC2* staining was observed, with no overlapping *MLC1v* expression. This can be assumed to be presumptive atrial cardiac tissue (figure 3.8b). Such staining was also apparent in late stage 45 conjugates, indicating that both ventricular and atrial chambers are present. This provides evidence for more complex cardiogenesis occurring in conjugates, exhibiting at least rudimentary A-P diversification or patterning.

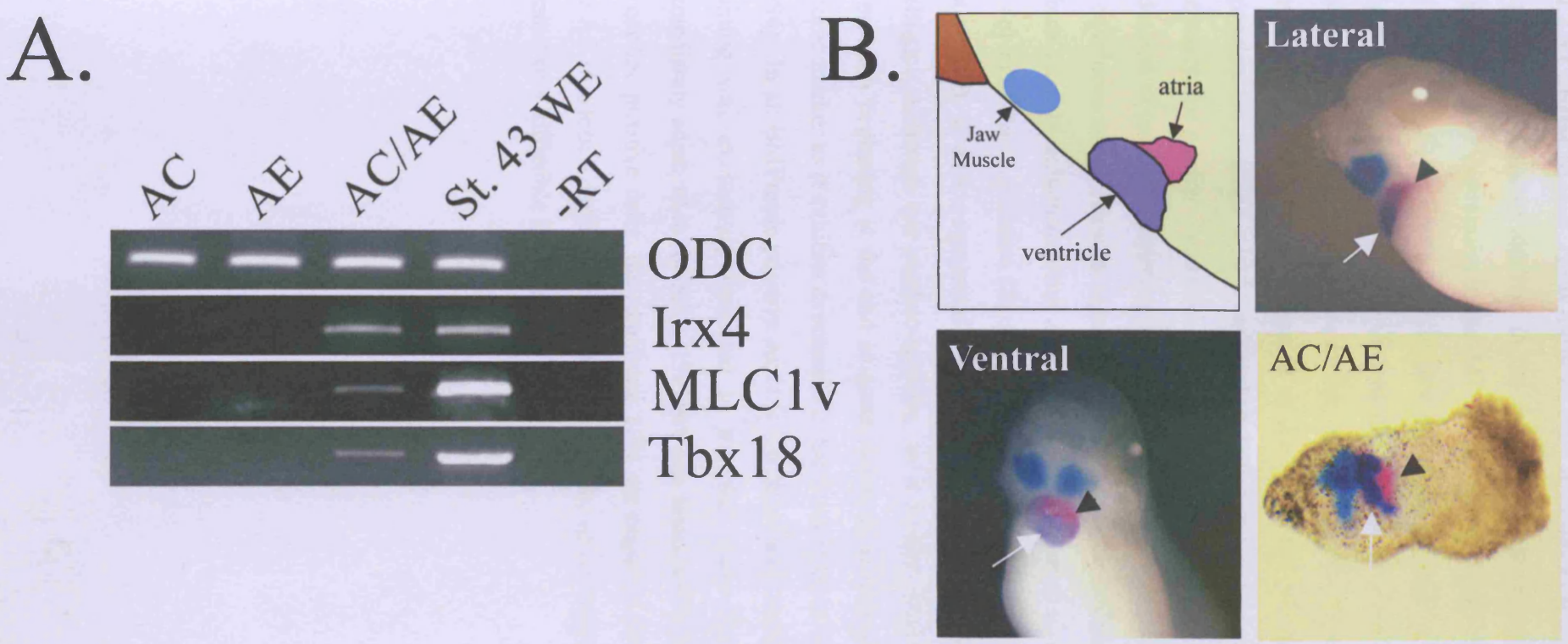


Figure 3.8 – AC/AE conjugates express regionally restricted cardiac markers at late developmental stages

[A] Conjugates (AC/AE) were cultured until stage 43 and analysed for late cardiac differentiation markers. They show robust expression of *Irx4, MLC1v, Tbx18,* compared to the anterior endoderm (AE) and animal cap (AC). **[B]** Overlapping double WMISH analysis was carried out to investigate regionally restricted cardiac gene expression. Stage 45 whole embryos were stained for the ventricular marker ***MLC1v*** and the pan-myocardial marker ***MLC2***. **Overlapping expression** demarcates ventricle (white arrow), whereas distinct **atrial** (black arrowhead) regions can be observed. Lateral and ventral regions of the whole embryos show both ventricular and atrial chambers. Similarly, stage 45 AC/AE exhibit similar staining patterns, indicating that more complex cellular diversification has occurred.

3.2.2.3 Cardiac Mesoderm is induced only in cells of the responder

Expression of cardiac markers in the AC/AE is a direct result of conjugation, given the absence of their expression in the AC or AE. Even though in this model it is assumed that the inducer (AE) produces an inductive signal that is interpreted by the responder (AC), the origin of cardiac tissue in AC/AE was not clear from RT-PCR analysis. To demonstrate that the AE induces cardiac fate in the responding AC, lineage tracing and WMISH was performed (figure 3.9). Conjugation of the AE to the AC has been shown to result in the formation of anterior structures, and it has previously been suggested that the AE is necessary for cardiac induction. Here it is shown that the AE is directly responsible for induction of cardiomyocytes in the stem-cell like AC cells, which are capable of responding to developmentally relevant signals to adopt numerous cellular fates. Conjugates exhibited robust cardiac induction, with approximately 60% of conjugates showing *Cardiac Actin* (*CAc*) and *CTnI* expression (figure 3.9) and 74% (n=31) showing *MLC2* positive cells (data not shown), at a developmentally relevant time that these genes are expressed in control siblings. Although not cardiac-specific, as it is also expressed in the somites with its expression beginning at the end of gastrulation in sarcomeric actin, *CAc* is also a useful cardiac marker as it exhibits expression in the heart from as early as stage 28 (Mohun *et al.*, 1984). In all BMPurple positive AC/AE (i.e. those expressing markers of cardiomyocytes) staining was exclusively restricted to the AC, most frequently in a single location immediately adjacent to the AE (71% have one focus and 11% have two closely linked foci of cardiac positive cells; the remaining 18% are negative [n=74]). Hence the AE induces the AC to adopt a cardiac fate upon conjugation, which maybe achieved by either cell-cell contact or a diffusible factor(s).

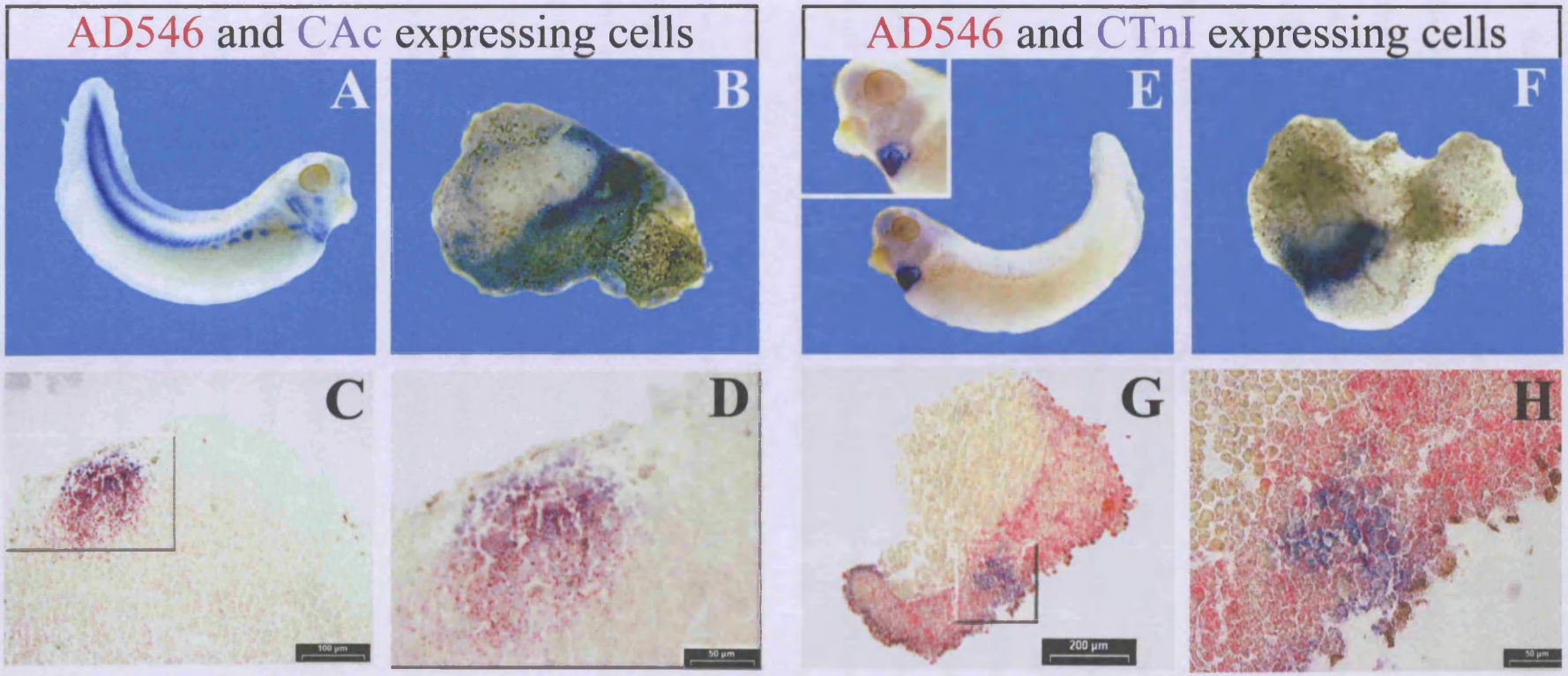


Figure 3.9 – The Anterior Endoderm shows robust induction of late cardiac differentiation markers that are restricted to the cells of the animal cap

One-cell stage embryos were injected with biotinylated dextran, from which animal cap were isolated to make conjugates with anterior endoderm and cultured until stage 34, when they were fixed and analysed by WMISH for the cardiac markers **[A-D]** Cardiac Actin and **[E-H]** Cardiac Troponin. Stage 34 whole embryo controls are shown (A, E). Staining with BMPurple revealed 63% (n=21) and 65% (n=23) of conjugates **[B, F]** showed CAc and CTnI expression, respectively. Subsequently conjugates were embedded in paraffin and sectioned, and lineage tracer was revealed by Fast Red substrate for avidin-coupled alkaline phosphatase **[C, G]**. Higher magnification of embossed area is shown **[D, H]**. Analysis reveals that cardiac positive cells are restricted to cells of the animal cap.

3.2.2.4 Cardiogenesis in AC/AE occurs in the same temporal pattern as the embryo

In the whole embryo, cardiac specification, determination, and terminal differentiation occurs in a defined sequence of events (section 1.2.2; Lohr and Yost, 2000). As described, the understanding of the exact sequence of events leading to the formation of differentiated cardiac tissue is poorly described. There is a significant length of time between specification of precursors and expression of markers of terminal differentiation and it is unknown when pre-cardiac cells are committed to form cardiac tissue (Mohun *et al.*, 2003). However certain key factors are known to be involved at different stages. The pre-cardiac mesodermal marker *Nkx2.5* is known to be expressed during early neurula stages in vertebrate species (Evans, 1999) Similarly, the T-box transcription factor *Tbx5* is thought to be essential for cardiac development and is expressed as early as stage 17 in the early heart field posterior to the expression domain of *Nkx2.5* (Horb and Thomsen, 1999). Lastly, the onset of terminal differentiation marker expression, although with some slight variation, occurs from approximately stage 28 (Chambers *et al.*, 1994; Drysdale *et al.*, 1994; Logan and Mohun, 1993). A recent report however contradicted this evidence. Work by Afouda and colleagues investigated the link between canonical and non-canonical Wnt signalling upon cardiogenesis, and showed that upon injection of GATA4 into AC MHCα expression was observed at stage 11 and stage 20 as a marker of cardiac specification (Afouda *et al.*, 2008). This is inconsistent with previous findings as *MHCα* is regarded as a marker of terminal differentiation (Logan and Mohun, 1993). In addition it has also been previously demonstrated that the induction of cardiogenesis by GATA4 generates faithful recapitulation of the events in vivo. This was examined, both by extensive PCR analysis of *MHCα* during development and also its expression upon injection of GATA4. Consistent with previous findings our results show that *MHCα* is indeed a cardiac specific terminal differentiation marker (Appendix I).

It has been shown that the AC/AE model shows expression of all aforementioned cardiac markers, and although there is some indication, it was unknown whether the temporal pattern of expression is identical to that of the whole embryo. To confirm that the AC/AE model for cardiac fate is a faithful recapitulation of the events in *vivo*, the temporal expression of known markers of cardiac fate at different stages of development of the AC/AE explants was analysed and compared to sibling control embryos (figure 3.10). *Nkx2.5* is first expressed in AC/AE conjugates at stage 18, with its expression maintained throughout cultivation. Similarly *Tbx5* has detectable expression levels at stage 18 and 24,

which peaks towards early tadpole stages. Importantly however, the terminal cardiac differentiation markers *MLC2* and *MHCα* are only detectable from stage 27 onwards. Therefore the expression of all these markers follows a similar temporal pattern to the corresponding sibling embryos and follows expression patterns previously described.

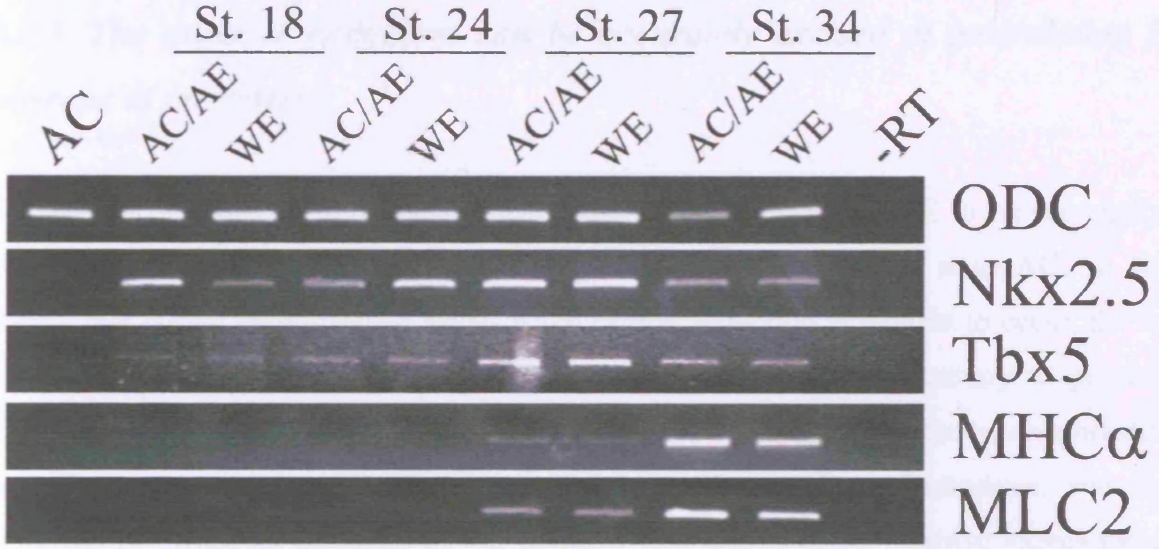


Figure 3.10 – Temporal expression of cardiac markers in AC/AE explants

Conjugates (AC/AE) of stage 10.25 anterior endoderm and stage 8.5 animal caps (AC) were cultured until different developmental stages. AC/AE explants and sibling control embryos (WE) were collected at stages 18, 24, 27 and 34 and analysed for cardiac markers shown. ODC was used as the loading control

3.4 Summary

3.4.1 The anterior endoderm can be accurately excised at gastrulation in absence of mesoderm

The results presented in this chapter demonstrate the ability of the AE to induce cardiac tissue in a naïve population of cells, namely the pluripotent blastula stage AC. It was shown that during early gastrula stages when cardiac induction is thought to occur, the AE could accurately and reproducibly be isolated from the embryo. Contrary to previous findings, it was conclusively shown that these explants of AE were free from contaminating mesoderm as ascertained by the absence of expression of *Xbra* (mesoderm) and *Gsc* (Organiser). Anterior character of AE explants was confirmed by positive expression of *Hex* and *Cerberus*, known to be required for anterior patterning. Upon establishing the assay, it was shown that the AE can act as an inducing source by its ability to confer anterior character in complex explants of marginal zone and induce expression of cement glands in ectodermal explants.

3.4.2 The AE is sufficient to induce cardiac tissue in the AC

It was investigated whether the excised AE was sufficient to induce cardiac tissue in the AC, previously shown to be competent to adopt cardiac fate. Upon conjugation of these two tissues (AC/AE), robust expression of cardiac markers was observed. When analysed at early stages, positive expression of the early cardiac transcriptional regulators *Nkx2.5* and *Tbx5* was observed. At later stages, strong expression of a variety of terminal cardiac differentiation markers (*CTnI, MHCα, MLC2*) was observed. Importantly, when analysed at specific developmental stages it was shown that expression of cardiac markers occurred in the same temporal manner as that of the whole embryo. Furthermore, when cultivated until later tadpole stages rhythmical beating tissue was observed in a number of conjugates. Analysis for expression of late regional specific markers revealed that the cardiac tissue induced in conjugates was also patterned to a limited extent. Strong expression of late ventricular markers *Irx4* and *MLC1v* was observed, with indication of atrial specific tissue revealed by overlapping expression analysis of *MLC2* and *MLC1v* transcripts.

By performing WMISH and lineage tracer analysis, the cell autonomy of cardiac marker expression was examined. Upon doing so, it was found that terminal differentiation markers were exclusively induced in cells of the responder. Taken together with the findings that neither the AE nor AC express mesodermal markers in isolation, this is positive confirmation that the AE is inducing cardiac fate in the responder. From these results it therefore has been unequivocally shown that the AE is necessary and sufficient to induce cardiac fate. Unclear at this stage however was the directness by which the AE induced cardiac fate; it was unknown whether the AE was directly inducing cardiac tissue or indirectly as result of induction of some other tissue. This question was later investigated by analysing the effect of blocking the expression of endodermal and mesodermal genes shown also to be expressed in the AC responder. Upon doing so, no obvious effects on cardiac marker expression were observed and therefore the role of the AE is direct (section 4.2.3). Therefore, by using pluripotent AC as a responder re-directing these cells toward a fate that they do not normally form is a true model of specification. Given that AC/AE conjugates faithfully reproduce virtually every aspect of early cardiogenesis, they are a tractable model for detailed analysis of cardiac induction.

CHAPTER 4 – SPECIFICITY AND COMPETENCE OF CARDIOGENESIS IN CONJUGATES

4.0 SPECIFICITY AND COMPETENCE OF CARDIOGENESIS IN CONJUGATES

4.1 Introduction

In chapter 3 it has been shown that the AE can accurately be excised from the embryo at gastrula stages in the absence of mesodermal tissue, and that this tissue can act as a signalling centre. Conjugation of AE to AC results in robust induction of cardiac fate restricted to the cells of the responder in the same temporal pattern as observed in the embryo. This demonstrates that the AE is sufficient to induce cardiac fate in a pluripotent responder. From studies of early embryonic development it is known that the early inductive event that directs cells to adopt a cardiac fate occurs some time during gastrulation. Removal of prospective heart-forming regions before stage 10.5 blocks heart formation but after stage 11 has no effect (Sater and Jacobson, 1990b). However, the precise details of the timing and the nature of the signals are unknown. This may be partly attributed to the retrospective analysis of cardiac differentiation genes as an indication of specification, which may not be a true reflection of its induction. In addition, low resolution analysis using beating structures as a readout did not provide accurate estimations of timing (Nascone and Mercola, 1995; Sater and Jacobson, 1989; Sater and Jacobson, 1990b). Currently, there are no markers specific for cardiac fate permitting them to be followed from the time of cardiac cell specification until terminal differentiation. Directing naïve AC cells toward a cardiac fate is a true *de novo* inductive event, with the AE providing significant signalling to direct and fairly comprehensively complete the cardiac program. The model therefore provided opportunity to address many of the unanswered questions of cardiac development

4.1.1 Chapter Aims

The aims of this chapter were to further characterise the AC/AE model of cardiogenesis. Having established that the AE is sufficient for cardiac specification and expression of

terminal cardiac differentiation markers, greater insight into how this occurred was required. It was therefore aimed to determine:

- The specificity of the AE in cardiac induction; is cardiac tissue induced directly or as a consequence of general mesendodermal induction?

- The timing at which cardiac precursors are specified; is cardiac specification by the AE dictated within a defined window of competence?

- The nature of the cardiogenic signal; is it secreted or mediated by cell-cell contact, and therefore does it act locally?

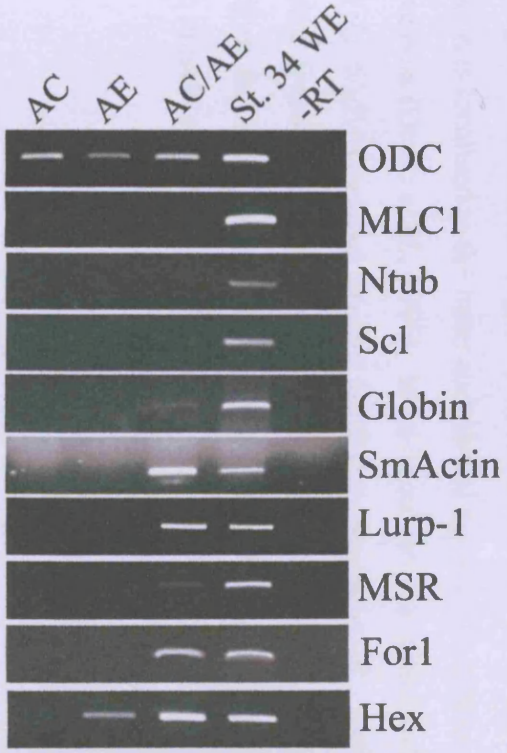
4.2 Results and Discussion

4.2.1 Specificity of induced cell fates by the AE

4.2.1.1 Anterior endoderm specifically induces cardiac cell fates

In previous classical experiments of mesoderm induction, a diverse range of cell fates were induced upon conjugation of the vegetal pole to the AC including notochord, muscle, blood and mesothelium (Nieuwkoop, 1969). Similarly, previous models of heart induction have shown that in addition to cardiac tissue, a variety of markers indicative of other cell fates are also induced. For example, Activin induction of cardiac tissue in AC results also in the expression of skeletal muscle tissue (Logan and Mohun, 1993), as well as a variety of other mesendodermal cell types (Green *et al.*, 1992). Conversely, GATA4 induction of cardiogenesis showed no induction of skeletal muscle but strong expression of a range of markers of endodermal and dorsal mesodermal cell types (Latinkic *et al.*, 2003). Hence, it was important to investigate whether the AE was specifically inducing cardiac fate in the responder, or whether it was just one of multiple cellular fates resulting from a more general induction of mesodermal and endodermal tissue in a similar manner to previously shown. To achieve this conjugates were made by the standard method and cultured until stage 34, and were then analysed for a variety of markers indicative of various cell types (figure 4.1).

A.



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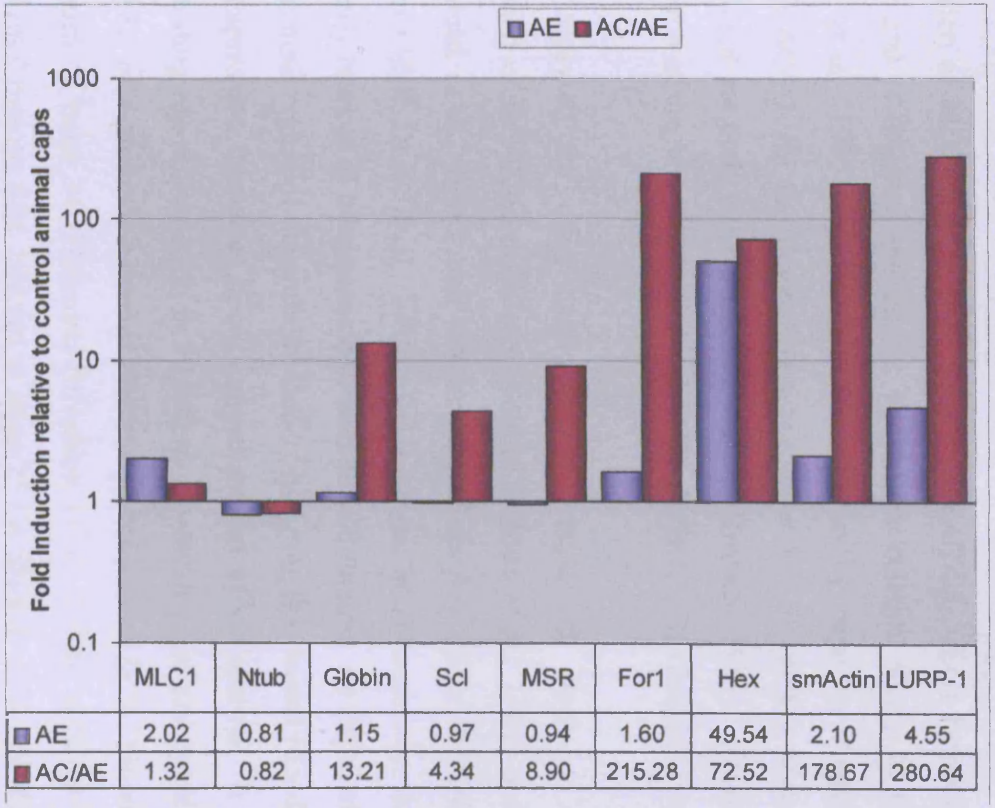


	MLC1	Ntub	Globin	Scl	MSR	For1	Hex	smActin	LURP-1
AE	2.02	0.81	1.15	0.97	0.94	1.60	49.54	2.10	4.55
AC/AE	1.32	0.82	13.21	4.34	8.90	215.28	72.52	178.67	280.64

Figure 4.1 – Anterior Endoderm specifically induces cell fates related to the cardiovascular system in absence of skeletal and neural tissue

[A] Conjugates (AC/AE) composed of stage 8.5 animal cap (AC) and stage 10.25 anterior endoderm, were cultured until control whole embryo siblings (WE) were at stage 34, and taken for analysis by RT-PCR. Stage 34 control AC and AE were also analysed. **[B]** ImageJ gel densitometry analysis of corresponding PCR

Stage 34 AC/AE showed no expression of skeletal muscle as shown by the lack of expression of *MLC1* (figure 4.1). Along with *MLC3*, *MLC1* forms the myosin of skeletal muscle and is detected toward the end of gastrulation solely in the somitic mesoderm (Theze *et al.*, 1995). This was further shown by negative immunostaining of cardiac positive AC/AE for the skeletal muscle marker 12/101 (n=43), a skeletal muscle specific monoclonal antibody (figure 4.2; Kintner and Brockes, 1984). Similarly, AC/AE show no expression of the neural specific marker *N-tubulin* (Ntub; Oschwald *et al.*, 1991).

When analysed for other cardiovascular related cell fates, AC/AE show moderate expression of the blood markers α-*globin* (Widmer *et al.*, 1981) and *Stem-cell leukaemia* (*Scl*; Mead *et al.*, 1998), and the endothelial marker *mesenchyme-associated serpentine receptor* (*MSR*; Devic *et al.*, 1996). *Scl* is a basic helix-loop-helix transcription factor that is an early marker of haematopoietic cells in vertebrates. Its expression begins at stage 15 in the ventral region of the embryo, closely mapping the ventral blood islands until stage 37 when expression decreases upon commencement of circulation. It is a key molecule in orchestrating blood formation in mesoderm downstream of embryonic patterning (Mead *et al.*, 1998). *αT4-globin* is a marker of erythrocytes located in the blood islands of the ventral mesoderm at stage 34 (Hemmati-Brivanlou *et al.*, 1990), with its expression in ventral mesodermal regions first detected at stage 25 (Walmsley *et al.*, 1994). *MSR* is a G-protein coupled receptor whose expression begins at gastrula stages in the mesoderm in all but dorsal regions. At tailbud stages however, it is a marker of the endothelial lineage and its expression is localised to the inner endothelial layer of procardiac tube and forming primary blood vessels (Devic *et al.*, 1996). In addition there is robust induction of the macrophage marker *Ly-6/uPAR-related protein* (*LURP-1*; Smith *et al.*, 2002), and also the vascular associated *Smooth Muscle α-actin* (*SmActin*; Warkman *et al.*, 2005), which is expressed throughout the heart from the onset of cardiac differentiation, but later restricted to the smooth muscle cells of the outflow tract.

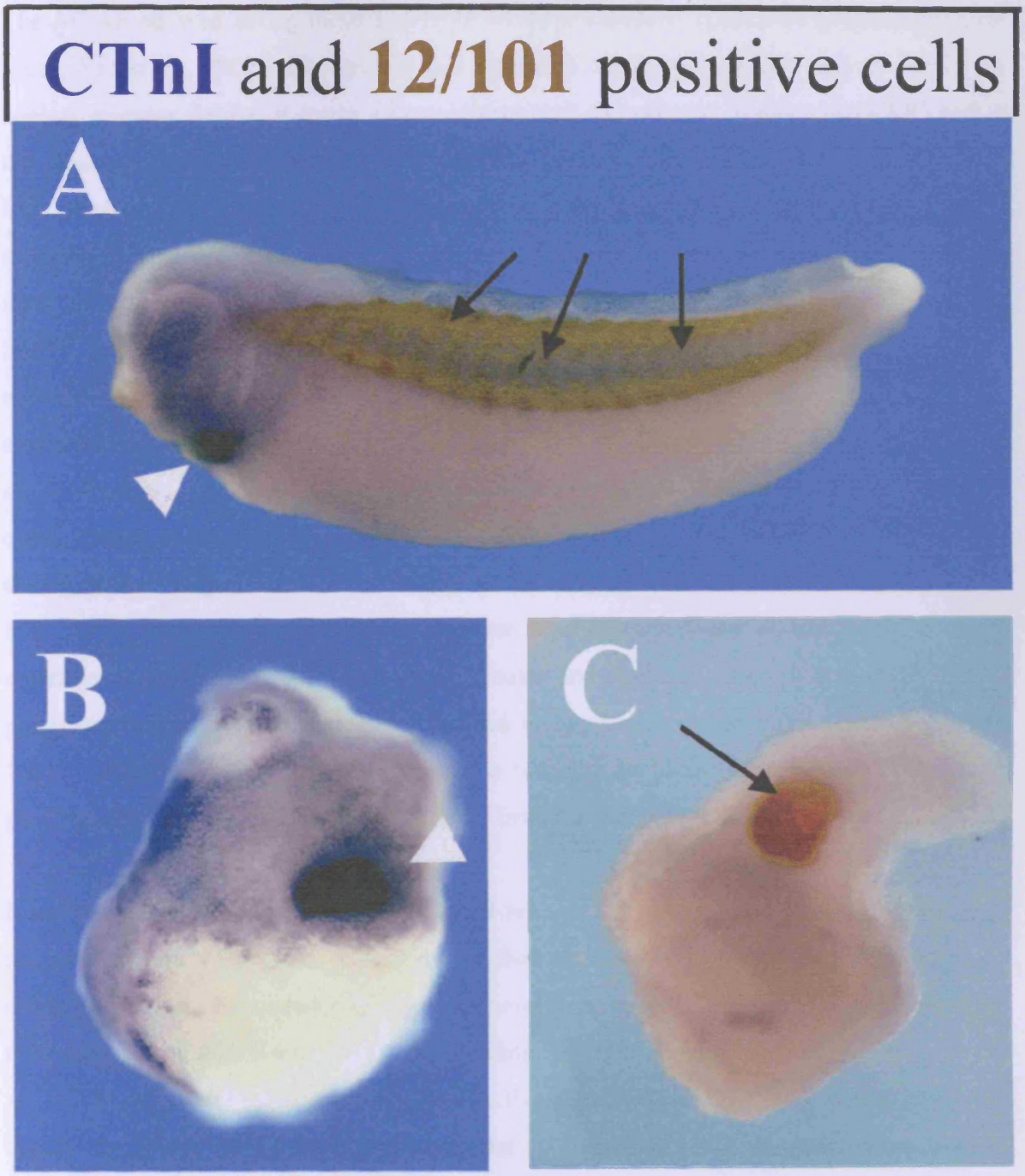


Figure 4.2 – AC/AE show negative staining for skeletal tissue

Analysis of *CTnI or CAc* expression by WMISH, followed by antibody staining with the skeletal specific monoclonal antibody 12/101. **[A]** Whole embryo (WE) controls show expression of *CTnI* in the heart region (white arrowhead), whereas 12/101 staining is restricted to the somites (black arrow). Analysis of *CAc* (n=15; not shown) and **[B]** CTnI (n=28) positive AC/AE showed no positive 12/101 expression. **[C]** As a positive control, animal cap treated with Activin, exhibiting elongation, show strong 12/101 expression.

Interestingly, the AC/AE exhibit induced expression of a subset of endodermal markers. The AC/AE showed strong induction of *farnesoid X receptor (FXR)-like Orphan Recep*tor (For1; Seo *et al.*, 2002), the *Xenopus* homologue of mammalian FXR. A member of the nuclear receptor family, it forms a heterodimer with the retinoid X receptor (RXR) and in the presence of ligand, binds DNA target sequences. It has been shown in mammals to bind phospholipid transfer protein, among others, an important protein involved in lipid metabolism (Urizar *et al.*, 2000). In *Xenopus*, *For1* expression begins at stage 31 and is restricted to the presumptive liver region, with peak expression at stage 35 (Seo *et al.*, 2002). Furthermore, the AE in isolation at stage 34 showed expression of *Hex,* which has already been shown to be expressed at stage 10 (figure 3.4). As previously mentioned, *Hex* expression begins at gastrula stages when it is uniquely expressed in the AE (Newman *et al.*, 1997; Zorn *et al.*, 1999) and this region is known to function as an anterior signalling centre (Jones *et al.*, 1999). Its expression then declines slightly and then increases again during tailbud stages. However, throughout development it is expressed in presumptive liver tissues with strong expression in the liver itself at later stages. Additionally, it shows extensive expression in the developing vascular endothelium, with strong expression in posterior cardinal veins, the dorsal aorta and endocardium of the heart (Newman *et al.*, 1997). Other endodermal markers were also found to be induced in the AC when it was possible to analyse the AC tissue in isolation from the AE (see section 4.2.3).

It has previously been suggested that vegetal regions are fated to give rise to endoderm by maternal factors. However, it was shown that regional specification, the expression of genes conferring commitment of tissue regions (Slack, 1991b) requires interaction with mesoderm (Horb and Slack, 2001). The finding that *Hex* is maintained in the AE but only AC/AE express *For1* (a gene restricted to the liver), led to investigation of whether this was due to the failure of AE to undergo regional specification. AE was excised at gastrula stages and cultured in isolation for analysis at different developmental time points. It was found that the AE express several known markers of the endoderm, namely *Endodermin* (*Edd*; Sasai *et al.*, 1996), *Sox17* (Hudson *et al.*, 1997), and *Hex* (figure 4.3a). All were expressed in the same temporal pattern as in the embryo, except for *Hex* whose expression was maintained throughout. This is likely due to the absence of a repressor in isolated AE that normally dampens *Hex* expression *in vivo*.

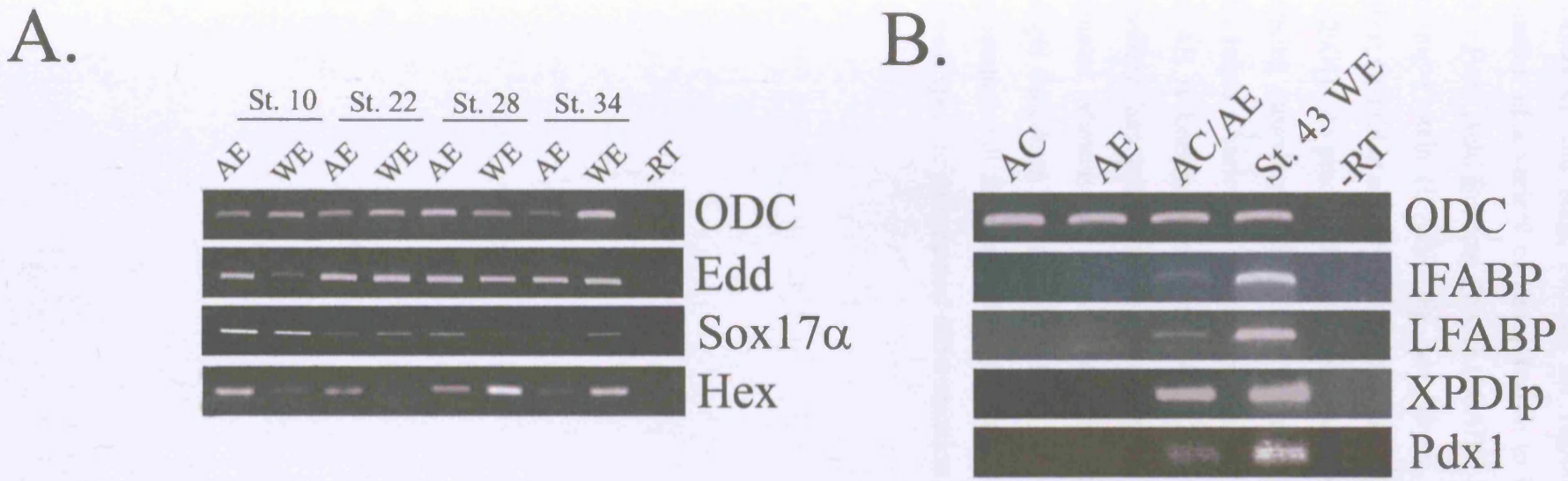


Figure 4.3 – Regional specification of the endoderm requires inducing signals from the mesoderm

[A] Anterior endoderm (AE) was excised at stage 10 and cultured in isolation shows expression of known endodermal markers; Endodermin, Sox17α, Hex.[B] AE was cultured until late tadpole stages and analysed for expression of regional specific markers of the liver (LFABP), intestine (IFABP), and pancreas (XPDIp, Pdx1). The AE shows no expression of such markers, which are however expressed in conjugates (AC/AE). Therefore, mesodermal derivatives induced in the AC are required to regionally specify the endoderm.

Analysis of late stage AC/AE for regional specific markers however showed robust induction of a variety of genes known to be specific to different tissues. This included Liver Fatty Acid Binding Protein (LFABP; Henry and Melton, 1998), Intestinal Fatty Acid Binding Protein (IFABP; Shi and Hayes, 1994), Pancreatic and duodenal homeobox 1 (Pdx1; Offield et al., 1996), and Pancreatic Disulphide Isomerase protein (XPDIp; Afelik et al., 2004). As predicted, these were absent from the AE alone. Therefore in AC/AE, AE is inducing mesodermal derivatives which signal back to regionally specify the endoderm. This forms a variety of foregut derivatives, such as intestine, liver, and pancreas, of which the AE is known in *vivo* to form (Zorn and Wells, 2009) This apparent reciprocal signalling has previously been suggested to be responsible for formation of liver in amniotes, where it was shown that cardiac mesoderm induces hepatic tissue in the adjacent foregut endoderm (Jung *et al.*, 1999; Rossi *et al.*, 2001). Further analysis revealed that endodermal cell fates are induced in the AC (section 4.2.3) but these never give rise to regional specific endodermal differentiation markers.

4.2.1.2 AC/AE show distinct differences from mesoderm induction in classic Nieuwkoop sandwich conjugates

The finding that AC/AE only expressed markers of restricted cellular fates (to those mainly of the cardiac and associated lineages) was unexpected. As already described, the AC/AE induction assay was based upon a modification of the Nieuwkoop mesoderm induction assay (Nieuwkoop, 1969). In many of these initial experiments into mesoderm induction, a range of different mesodermal cell fates were induced in the responding tissue. Hitherto, relatively little is known regarding the inductive capacity of the AE, other than its ability to induce anterior character as ascertained by its ability to induce cement glands (Jones *et al.*, 1999). However, in all previous conjugation studies the incidence of heart induction was either not investigated or poorly documented. In the experiments of Grunz and Tacke (1986), the nature of mesoderm inducing signals of conjugates across a filter were studied. They observed the formation of heart like structures, however, the incidence was very low (only 2%) and the results were not reproducible. It is likely that the discrepancies in levels/incidence of cardiac induction between previous models and those presented here may lie with obvious differences in experimental strategy. One possible explanation may be due to the differences in embryonic stages of the components of the conjugates in each of the two systems. AC/AE explants are heterochronic, composed of stage 8.5 AC and stage 10.25 AE. Nieuwkoop sandwiches however, are isochronic, consisting of both AC and vegetal pole from stage 8.5 embryos. To address if this was in fact the case, vegetal poles were isolated from stage 8.5 embryos as in the classic Nieuwkoop model, and aged until control sibling embryos reached stage 10.25 (i.e. the time at which routine AE excision occurs). These aged vegetal poles were then conjugated to stage 8.5 AC, and cultured until stage 34 for gene expression analysis, to allow comparison to normal Nieuwkoop sandwiches and AC/AE (figure 4.4).

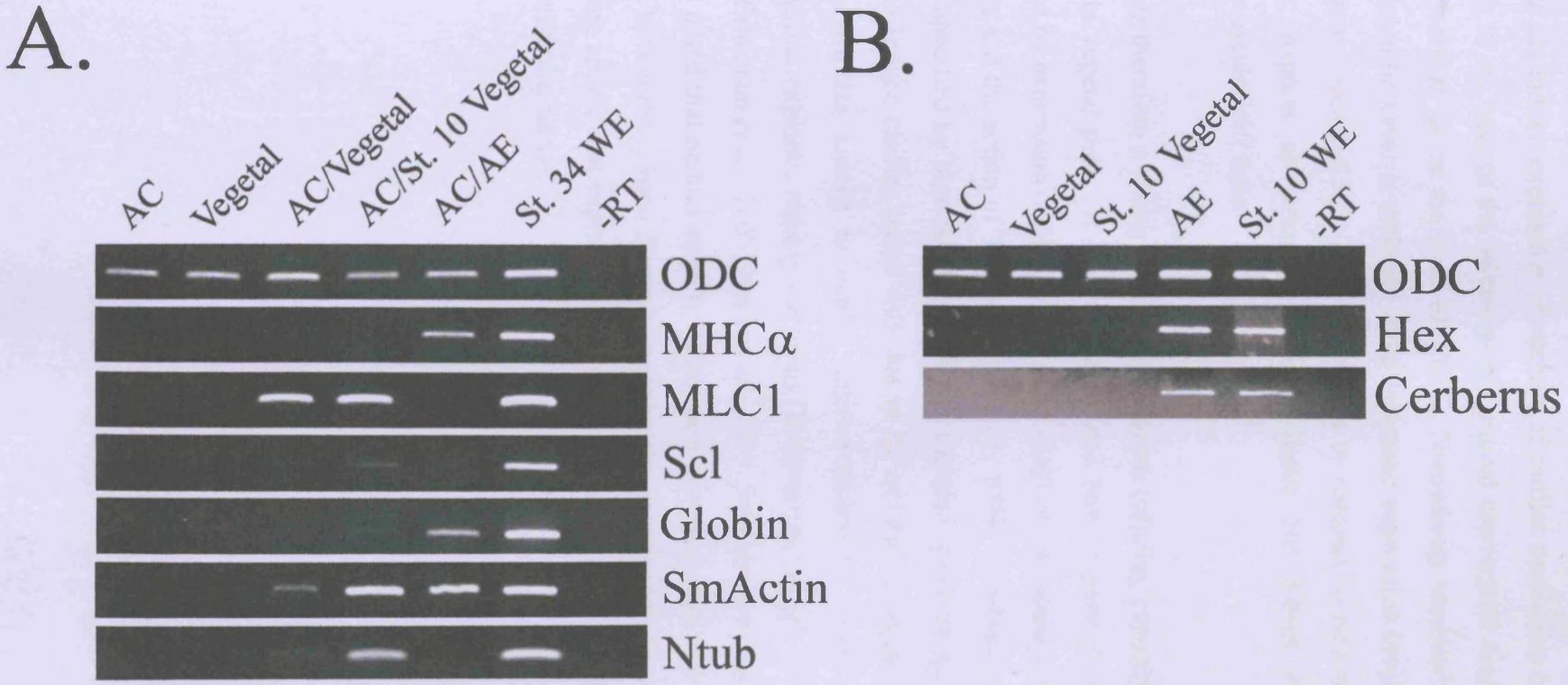


Figure 4.4 – Animal Cap/Vegetal Pole conjugates show robust expression of markers of skeletal and neural tissue but not of cardiac

[A] Stage 8.5 Vegetal poles were isolated, and either conjugated to stage 8.5 animal cap (AC) forming classic Nieuwkoop sandwiches (AC/vegetal), or aged to stage 10 and then conjugated (AC/St. 10 Vegetal). Control animal cap/anterior endoderm conjugates (AC/AE) of stage 10 AE and stage 8.5 AC were also made. Samples were taken when sibling embryos (WE) reached stage 34 and analysed by RT-PCR for the described markers. **[B]** Blastula stage vegetal poles and stage 10 vegetal poles (excised at stage 8) show no expression of *Hex* and *Cerberus*, in contrast to stage 10 AE.

As previously documented (Nieuwkoop, 1969; Sudarwati and Nieuwkoop, 1971), stage 8.5 animal cap/vegetal pole conjugates show strong induction of skeletal muscle. However, they do not exhibit expression of markers of cardiac mesoderm (*MHCα).* This was also the finding in the case of the heterochronic animal cap/vegetal conjugates, which express the same markers as in the case of classic Nieuwkoop sandwiches. The degree of gene expression however is different, with increased expression levels in heterochronic vegetal conjugates (figure 4.5). This however is in contrast to heterochronic AC/AE which as shown, express no neural or skeletal tissue but robust expression of markers of cardiovascular cell fates.

There is therefore a distinct difference between inducing capacity of the AE and that of the blastula vegetal pole. It has been described how at gastrula stages the AE is uniquely marked by expression of *Hex*, which is the result of combined action of dorsally enriched β-catenin and the action of TGFβs (Zorn *et al.*, 1999) Furthermore, it was reported that the AE is specified by blastula stages. It was therefore possible failure of the blastula vegetal pole to induce cardiac tissue was due to its inability to impart anterior character. As a result, markers known to confer anterior patterning were examined in vegetal and endodermal explants, namely *Cerberus* (Bouwmeester *et al.*, 1996; Silva *et al.*, 2003) and Hex (Brickman *et al.*, 2000; Jones *et al.*, 1999; Smithers and Jones, 2002). Upon doing so, it was found that neither stage 8 vegetal pole nor aged vegetal poles express either marker, which have already been shown to be robustly expressed in the AE (figure 4.4b). It would therefore appear that expression of anterior markers correlates with the ability of the AE to induce cardiac tissue.

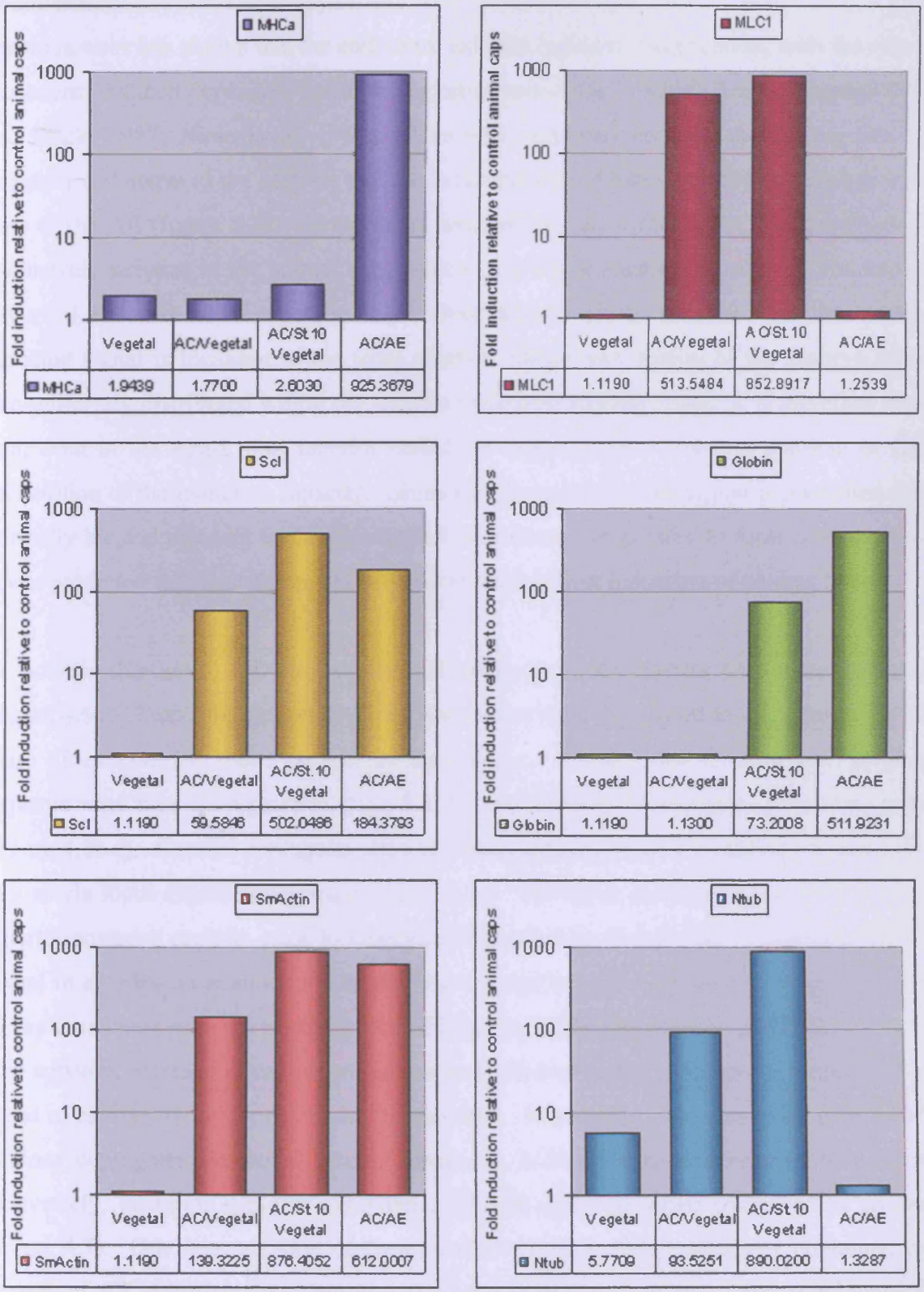


Figure 4.5 – Densitometry readings for various markers in iso- and hetero-chronic conjugates

4.2.1.3 Cardiac inducing capacity is restricted to a localised region of anterior endoderm

Previous work has shown that the endoderm exhibits regional specification, with the type of mesoderm induced dependent upon the region of endoderm to which it is conjugated (Dale and Slack, 1987; Nieuwkoop, 1969). This was supported here by the finding that the posterior endoderm of the gastrula embryo is not capable of inducing cardiac tissue as is the case of the AE (figure 3.7). Furthermore, induced cardiac tissue in AC/AE conjugates is exclusively induced in the animal cap, usually in a single location adjacent to the inducer (figure 3.9). All taken together, these observations clearly indicate that the cardiac-inducing signal is localised to the more anterior endodermal region of the embryo and is non-uniformly distributed within the anterior endoderm explant itself. It is therefore likely that, even in the small most anterior endoderm explants, there exists a gradient or finer localisation of the inductive capacity. Given that the most anterior region is presumed to be normally located adjacent to the dorsolateral mesoderm that is fated to form cardiac tissue, it was predicted that this region would result in more robust induction of cardiac fate.

To address this issue, AE was excised and subdivided into anterior and posterior halves (figure 4.6a). Two anterior and two posterior halves were then fused to compensate for the mass of endodermal tissue present in the inducer. These fused anterior and posterior explants were then conjugated to stage 8.5 AC and assayed for induction of cardiac tissue (figure 4.6b-f). Control conjugates showed robust expression of the cardiac marker *CTnI,* in a single focus expression domain (figure 4.6c). However, division of the AE into more anterior/posterior regions prior to conjugation resulted in enrichment of cardiac-inducing signal in anterior-most endoderm containing conjugates with very little activity remaining in explants made with the posterior part of inducing tissue (figure 4.6e and f respectively). The apparent increase in cardiogenesis can be attributed to the presence of more than one focus of cardiac tissue per conjugate (figure 4.6e). In addition, when analysed by RT-PCR anterior conjugates showed a 2-fold increase in *MHCα* expression relative to controls. Conversely, posterior explants exhibited a 25-fold decrease when compared to controls (figure 4.7). One interpretation of these results is that anterior conjugates contained two regions of AE, doubling the effective tissue with inductive capacity. Further evidence for the discrete action of cardiac inducing signal came from dissociation and re-aggregation of AE tissue, followed by conjugation to AC. As a result of this manipulation, cardiac tissue was induced in multiple small foci as opposed to one discrete focus of expression (Figure

4.6f), suggesting that cardiac inducer tissue acts locally and proportionately. In addition, this result suggests that the cardiac-inducing signal(s) are; (1) stably associated with the specific AE cells or (2) continuously produced as their action is not abolished by tissue dispersal, or a combination of both.

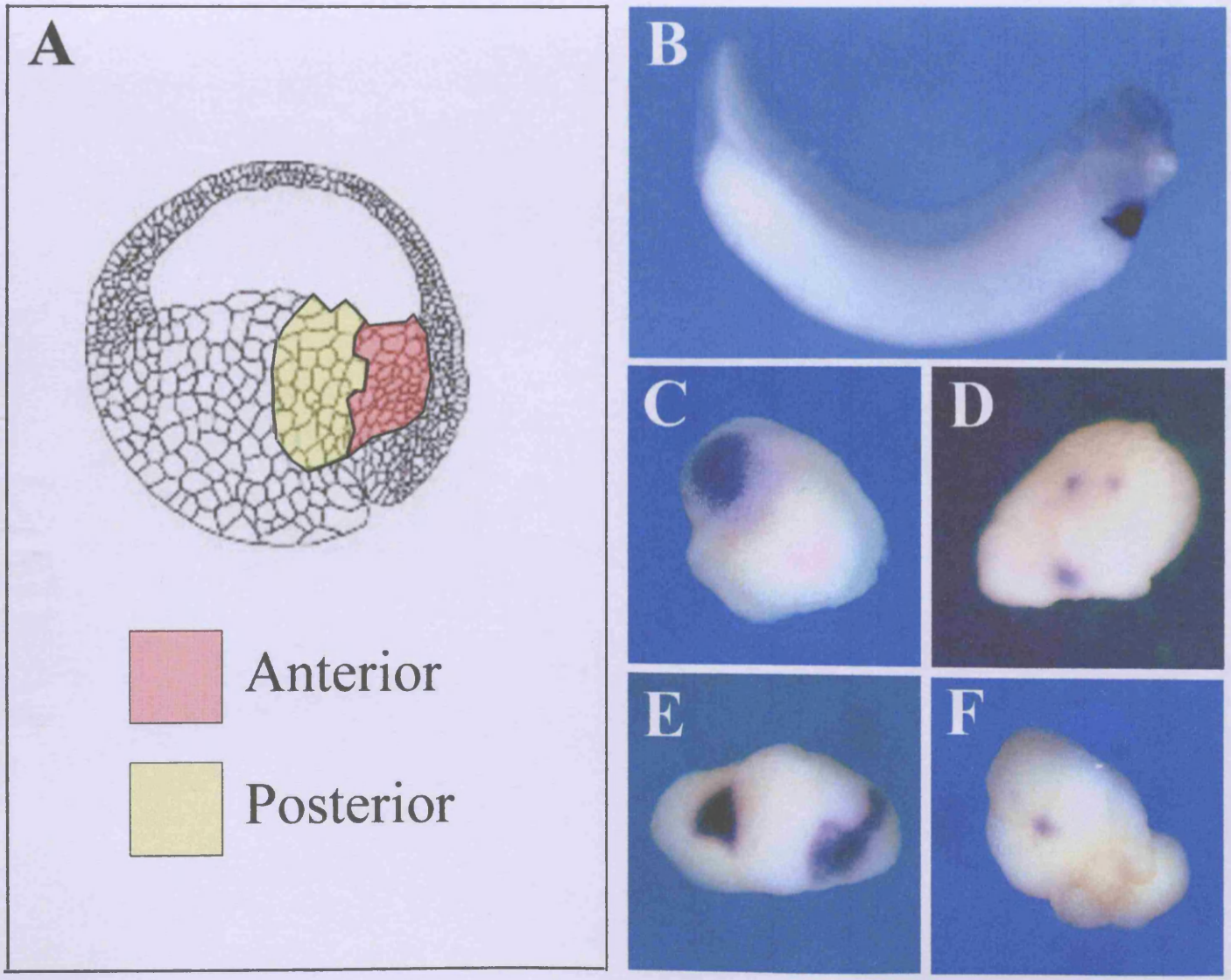


Figure 4.6 – Cardiac-Inducing Signal of the Anterior Endoderm is localised to the most anterior region

[A] Anterior Endoderm (AE) was excised from stage 10.25 whole embryo (WE) and dissected into anterior and posterior regions. Two anterior and two posterior halves were then fused to compensate for the mass of endodermal tissue present, to form anterior **[E]** and posterior **[F]** AE explants. These were conjugated to stage 8.5 animal cap (AC), as well as control conjugates (AC/AE; **[C]**), and cultured until stage 34 for *in situ* analysis. Samples of AE were also dissociated **[D]** in CMFM and conjugated to AC. **[B]** WE controls for the cardiac marker *CTnI*.

As described, excised AE shows strong expression of the homeobox transcription factor *Hex*, a gene whose expression demarcates the AE at stage 10 (Jones *et al.*, 1999; Zorn *et al.*, 1999), and was also found to be the case in conjugates of AC/AE (figure 3.4). It was predicted that the more anterior AE may express a proportionally greater level of *Hex* given the aim was to isolate the most anterior cells. Anterior/posterior fused endoderm samples were analysed upon excision for *Hex* expression (figure 4.7b). It was found that the more anterior AE shows 2-fold higher levels of *Hex* expression when compared to control AE, versus the posterior samples which show 50-fold reduction in expression. This therefore reflects an accurate excision and subdivision of the AE into more anterior and posterior regions, with increased cardiogenic potential of anterior fused conjugates closely correlating with the *Hex* expression domain.

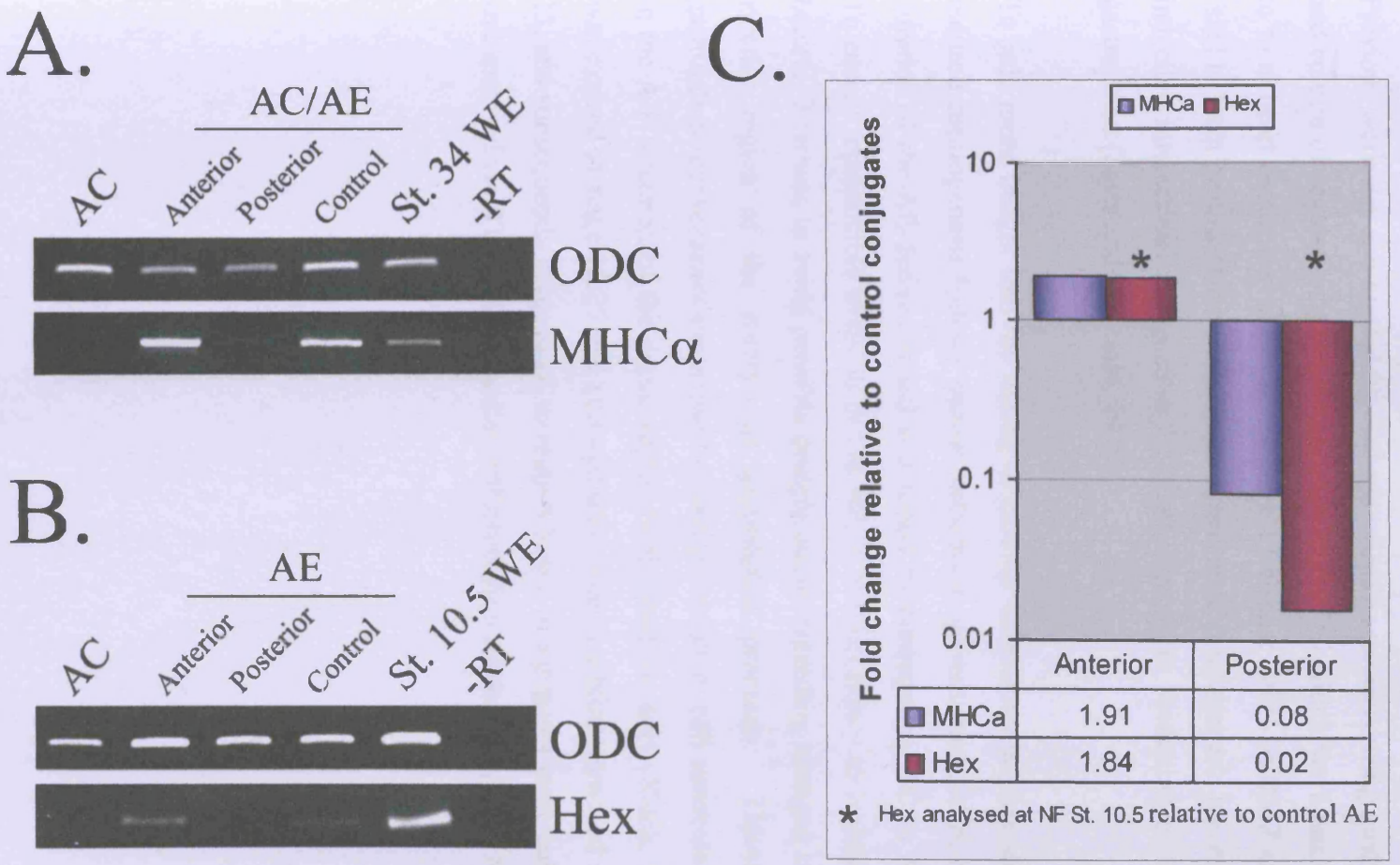


Figure 4.7 – Increased anterior character of fused AC/AE correlates with an increase in cardiac fate specification

[A] Conjugates were made with fused anterior or posterior halves of AE and analysed at stage 34 for cardiac marker expression. **[B]** Samples of control AE, fused anterior AE or fused posterior AE were also taken immediately after excision for analysis of *Hex* expression. **[C]** Gel readouts were quantified using ImageJ analysis.

4.2.2 Timing of Cardiac Specification in AC/AE Conjugates

4.2.2.1 Cardiac induction occurs during gastrula stages in AC/AE conjugates

Previous work has shown that cardiac specification occurs during gastrulation. Extirpation and culture of regions of prospective heart formation which were analysed for their ability to form heart tissue, revealed that by stage 13 almost 100% of DMZ explants were already fated to form beating tissue. Thus specification of cardiogenesis has already occurred, with inductive interactions responsible for heart mesoderm formation complete by the end of gastrulation (Sater and Jacobson, 1989).

To gain more insight into the timing of cardiac induction and determine whether AC/AE induced cardiogenesis follows similar patterns to general mesoderm inductive events, the duration of the AE-derived signal was tested by conjugating AC to AE of different ages. To ensure reproducible excision of the AE, it was necessary to isolate it at stage 10.25 as before. This was to avoid possible complications regarding changes in composition of the anterior region of the embryo as gastrulation proceeds. Through the process of gastrulation, *convergence-extension* movements result in cell intercalation with elongation in the A-P direction of the dorsal region of the embryo only (Slack, 1991a). Hence, AE was excised at stage 10.25 and cultured until control siblings reached stage 11.5 and stage 13, and subsequently conjugated to stage 8.5 AC. Conjugates were cultured until stage 34, and analysed via RT-PCR for cardiac differentiation markers (figure 4.8).

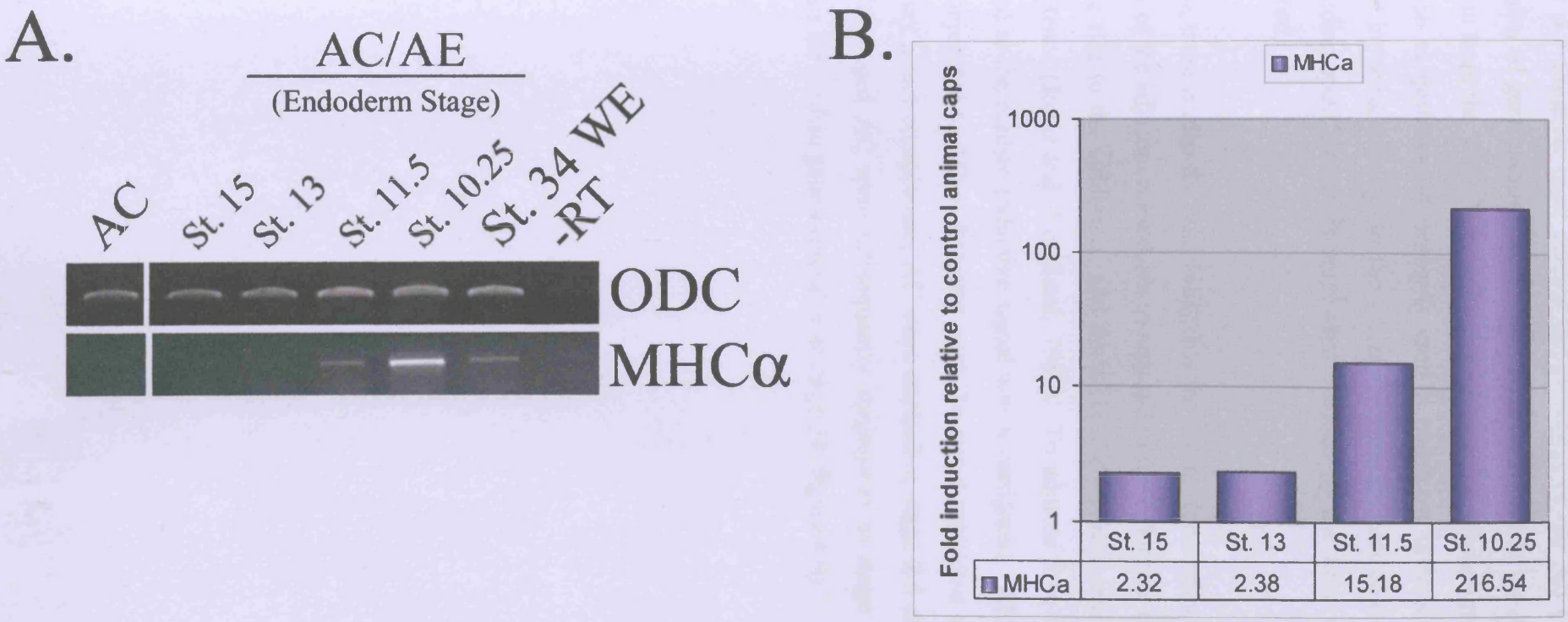


Figure 4.8 – The Anterior Endoderm-derived cardiac inducing signal is lost after gastrulation

[A] Samples of anterior endoderm (AE) were excised from stage 10.25 embryos and conjugates (AC/AE) were made between stage 8.5 animal caps (AC) and aged anterior endoderm explants as indicated. RT-PCR analysis was carried out at embryonic stage 34. **[B]** ImageJ gel densitometry analysis of corresponding PCR

Figure 4.8 suggests peak cardiac inductiveness of the AE (after stage 10) occurs at the beginning of gastrulation, when the AE is conjugated to the AC immediately following its excision from the WE. The levels of cardiac differentiation marker expression then rapidly decrease as gastrulation proceeds until it is almost undetectable at stage 13. Thus the cardiac inducing capacity of the AE is lost through gastrulation, and that the time-window for cardiac specification is somewhat dictated by the time which the inducible factor is produced.

In *vivo*, there is also the possibility that the timing for cardiac induction is restricted by the ability of the adjacent mesoderm to respond to the inductive signal. AC are normally fated to give rise to the epidermis, and therefore their pluripotency is only a short window of competence (Jones and Woodland, 1987). To address this, the competence of the AC to respond to the cardiac inductive signal was investigated. AE were excised at stage 10.25, and conjugated to AC of different ages. To avoid problems of altered composition of AC that may affect competence, AC were excised at stage 8.5 and kept open by culturing in LCMR. Aged AC were subsequently conjugated to stage 10.25 AE, and cultured for analysis for cardiac gene expression at stage 34 (figure 4.9).

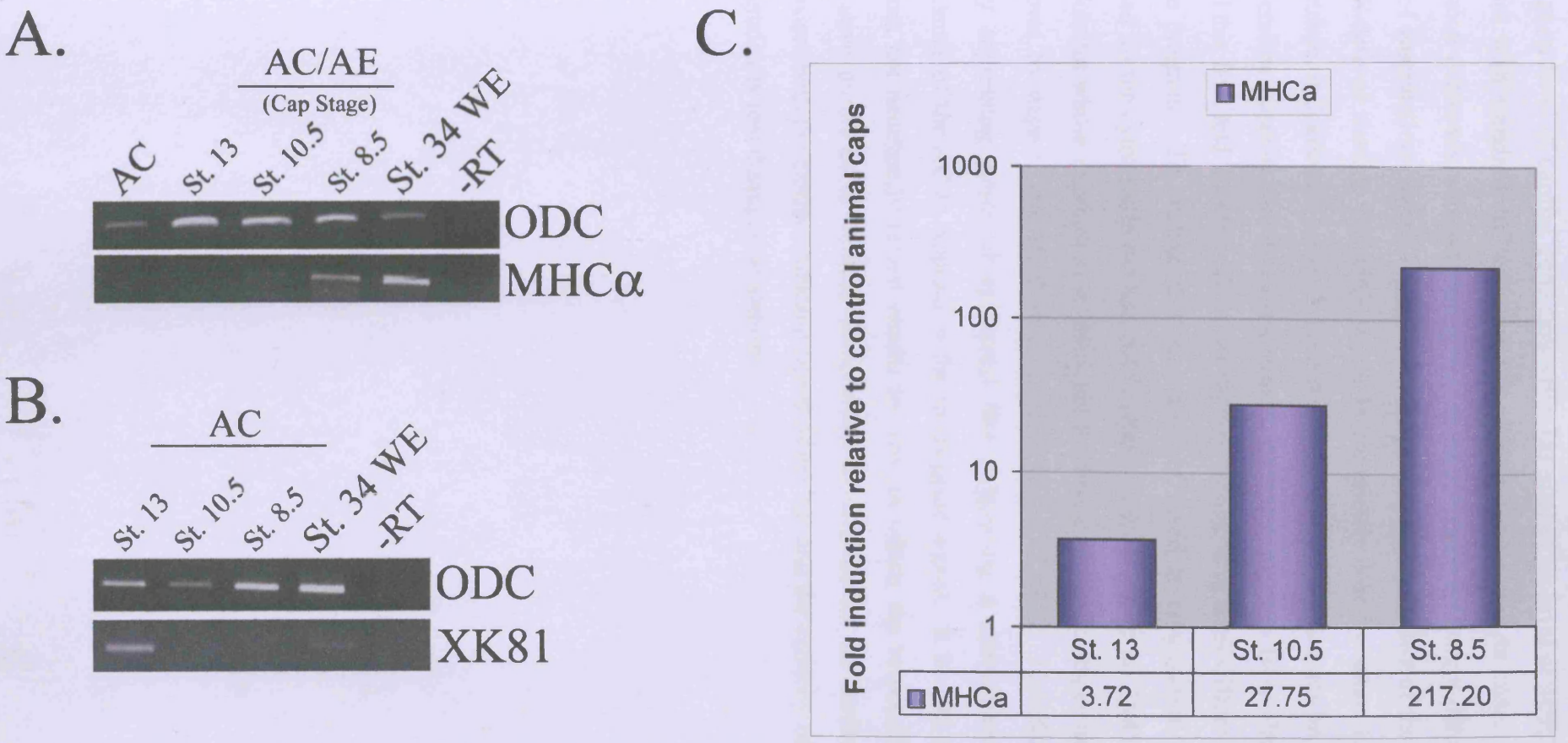


Figure 4.9 – Competence of animal cap cells to respond to cardiac fate-inducing signal is lost by the end of gastrulation

Prior to conjugation to stage 10.25 Anterior Endoderm (AE), animal caps (AC) were isolated at stage 8.5 and aged in LCMR to the stages indicated. **[A]** Conjugates (AC/AE) were cultured until stage 34 for cardiac marker gene expression. **[B]** Samples of aged AC were also taken immediately for analysis of the cytoskeletal marker *XK81*. **[C]** ImageJ analysis shown.

The highest level of cardiac gene expression (as marked by level of *MHCα* expression) was apparent with standard AC of stage 8.5. Upon conjugation to older AC, cardiac gene expression decreases with increasing age of AC, with levels barely detectable beyond the onset of gastrulation (figure 4.9). It would appear that the ability of the AC to respond to the AE-derived cardiac inducing signal is completely lost by stage 13. In addition to competence, it is known that by default the AC autonomously adopts an epidermal fate. It was therefore possible that by conjugating to such late AC they have already been directed toward this fate, and thus the AE is not capable of directing trans-differentiation toward the cardiac program. To analyse this, the aged AC used in conjugation were immediately analysed for the cytokeratin marker, *XK81* (figure 4.9b; Jonas et al., 1985). *XK81* is a Type I cytokeratin whose expression is restricted to early embryonic stages in epidermal layers. As shown, by stage 13 the AC show robust expression of *XK81*. Therefore stage 13 AC are already expressing genes of epidermal fate suggesting a mechanism for the loss of competence of the AC to respond to the cardiogenic signal. It should be noted however, this does not necessarily reflect results in *vivo,* in which the responding MZ may have alternsative durations of cellular competence to respond to the cardiogenic signal. In conclusion, both the cardiac inducing signal of the AE and the capacity of AC to respond to it are gradually lost during gastrulation.

4.2.2.2 Cardiac inducing signal requires 1-2 hours to specify cardiac fate

It has been shown that conjugation of gastrula AE to blastula AC results in robust expression of terminal cardiac differentiation markers (figure 3.8). Furthermore, the specification events must occur during early gastrulation, in terms of inductive capacity and responding competence. Due to poor characterisation of markers of cardiogenesis and the lack of early cardiac-specific genes, the results thus far have relied upon expression of terminal differentiation markers to signify specification events. As mentioned, there is significant time between specification and expression of cardiac differentiation markers (Mohun and Sparrow, 1997). In the context of the AC/AE conjugation model, it was therefore unknown whether the AE role was solely involved in the specification events or was required for further commitment and differentiation. It has also already been shown that it is likely that underlying AE is required later for more complex morphogenetic cardiac development (figure 3.9; Muslin and Williams, 1991; Sater and Jacobson, 1989; Tonegawa et al., 1996).

It was therefore possible that the AE may have either a transient or more sustained role in cardiogenesis. Hence, the minimum contact time between AE and AC was established. In order to accomplish this, the conjugation assay was modified to allow timed exposure of animal cap to the AE. This was first investigated by means of a trans-filter assay, in which AC and AE were separated by a filter membrane. Previous work has utilised transfilter experiments in induction assays generally to determine the nature of the inducing signal (cell-cell contact *versus* diffusible factors). For example, in the case of mesoderm induction as a result of vegetal-animal pole conjugation, results were unclear as to whether this was due to cell-cell contact. Experiments by Grunz and Tacke (1986) in which conjugation was carried out across a 0.4 μM nucleopore filter, still showed induction of mesodermal cell fates. Electron Microscope analysis of the transfilter conjugates showed no cellular protrusions could traverse the membrane and therefore induction was the result of diffusible factors. Further refinement by Gurdon (1989) however, revealed that the level of induction across the filter is dampened compared to control conjugates and although not requiring cell contact, the response is limited to cells within a few cell-diameters.

To perform similar analyses to the AC/AE model, AE was excised as normal and conjugated to AC across a 0.4 μM filter, shown to not permit cell-cell contact (Grunz and Tacke, 1986; Gurdon, 1989) in an experimental setup previously described (figure 4.10a;

Slack, 1991a). The experiment revealed that conjugation of AC to AE across this filter in which cell-cell contact is not permitted, was still sufficient to result in specification of cardiac fate and later expression of terminal differentiation markers. From this it can be concluded that the cardiogenic signal from the AE is therefore diffusible as the barrier between the inducer and responder did not abrogate cardiac induction.

However, it proved very difficult to generate stable trans-filter AC/AE conjugates that provide uniform exposure of AC to AE-derived signals. Therefore, an alternative assay for timed exposure of AC to AE-derived signal was developed, in which the conjugated AC was peeled from AE after a defined length of culture (subsequently named 'peeled animal cap assay', figure 4.10b). The basic design of this assay was described by Gurdon and colleagues in their studies of muscle induction (Gurdon *et al.*, 1985). In these experiments to determine the duration of contact required for muscle induction in conjugates of animal and vegetal poles, conjugates were made as usual and after a defined length of time separated to form so-called 'exconjugates'. To ensure accurate separation of tissues Gurdon *et al.* (1985) took advantage of visual differences between AC and larger vegetal cells, and also labelled cells of the vegetal pole with radioactive serum albumin. Therefore accurate separation was confirmed by lack of radioactivity in cells of the AC.

This method was modified by labelling AE at the 2-cell stage with rhodamine-dextran, and it was excised at stage 10.25 for conjugation with stage 8.5 AC. AC were peeled away under fluorescent light after a defined period of time, to ensure that no AE cells remained on AC explants after manipulation (figure 4.10b). Peeled animal caps (pAC) were cultured until control siblings reached stage 34 for analysis of cardiac markers expression (figure 4.10c).

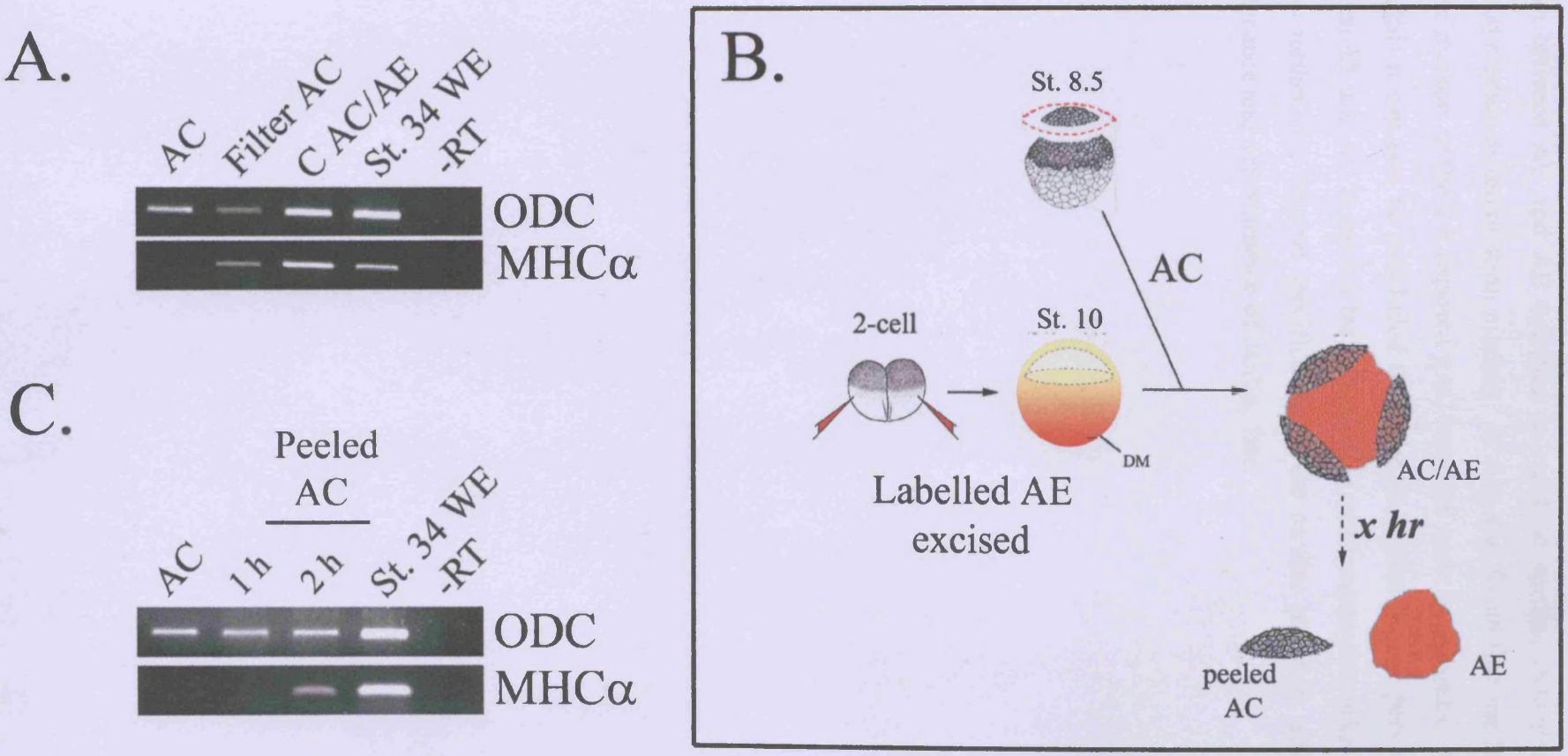


Figure 4.10 – Cardiac induction by the anterior endoderm is achieved after 2 hours of conjugation by a diffusible signal

[A] Anterior endoderm (AE) was excised from stage 10.25 and conjugated with stage 8.5 AC across a 0.4 μM filter. Samples were cultured until stage 34 for RT-PCR analysis. **[B]** Schematic of peeled animal cap assay. Vegetal cells of 2-cell embryos were injected with rhodamine-dextran to label AE, excised at stage 10.25. Conjugates were made with stage 8.5 AC, and caps removed after a defined period of time (1 or 2 h) under fluorescence to ensure complete removal of AE. **[C]** Peeled AC (pAC) were then cultured until stage 34 for analysis of cardiac marker expression.

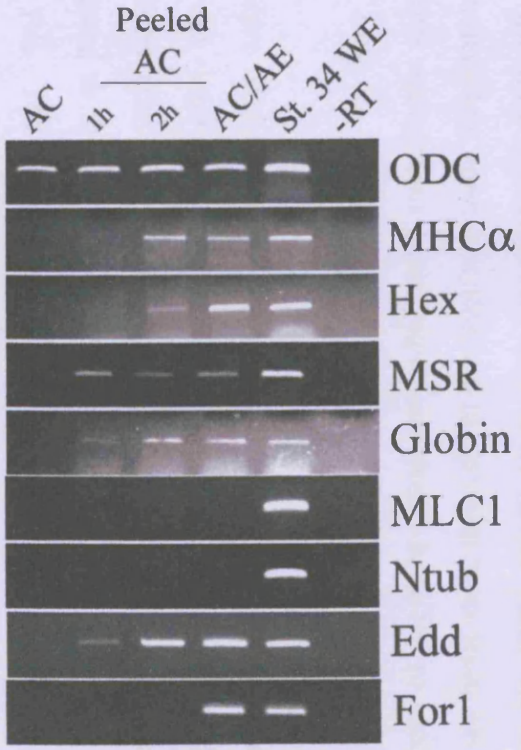
Using this assay it was found that only after 2 h (but not 1 h) had a sufficient period of contact between AC and AE occurred to result in cardiac induction and expression of terminal cardiac differentiation markers. It was also found that the level of expression of cardiac markers in the 2 h exposed pAC were the same as in control AC/AE (figure 4.11). From this it can also be concluded that after the initial 2 hour period, no further contact between AE and AC is needed for induction of cardiomyocyte markers. Therefore the AE has no further role beyond specification of the cardiac program, and is not required for maintenance and determination of cardiac fate.

4.2.3 Molecular Characterisation of Peeled Animal Caps

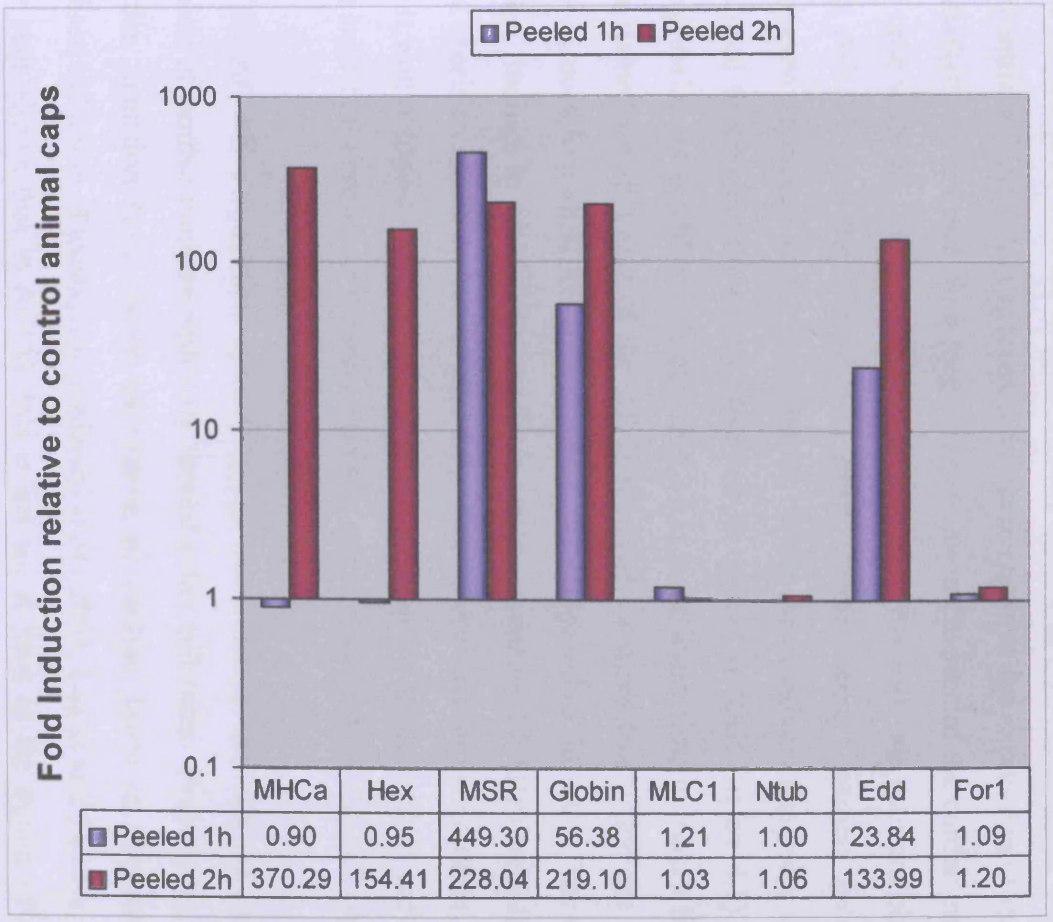
4.2.3.1 Cellular fates induced in the AC by the AE

The positive finding that AC cells peeled from the surface of the AE show expression of terminal cardiac differentiation markers, after 2 h contact, revealed sufficient time had elapsed to allow specification of cardiac precursors to the extent that they are able to become determined. These results also revealed continued involvement of the AE beyond specification, at least with regards to expression of certain terminal differentiation markers, is not required. As this study was focussed toward cardiac specification, this more simplistic model of AC/AE permitted further in depth analysis of the signalling involved in cardiac induction. Furthermore in normal control AC/AE, induced cardiomyocytes in the AC were still in association with the AE. Although it was determined that the markers of various cell fates were the result of conjugation, as they were not expressed in samples of AE alone, it was unknown whether they were expressed in the AC or the AE (figure 4.1). In the case of cardiomyocytes, it was shown that cardiac cell fate occurred in the cells of the AC (figure 3.9). It was next addressed whether this was also the case for the peeled AC, and whether 2 h of contact is also sufficient to induce the other cell fates observed upon conjugation (figure 4.11).

A.



B.



	MHCa	Hex	MSR	Globin	MLC1	Ntub	Edd	For1
Peeled 1h	0.90	0.95	449.30	56.38	1.21	1.00	23.84	1.09
Peeled 2h	370.29	154.41	228.04	219.10	1.03	1.06	133.99	1.20

Figure 4.11 – The profile of cellular fates induced in peeled animal cap after 2 hours of exposure is indistinguishable from AC/AE conjugates

[A] Rhodamine-dextran labelled anterior endoderm (AE) was excised from stage 10.25 embryos and conjugated to stage 8.5 AC. The AC were then peeled away after 1 or 2 h, and the rhodamine-negative caps then cultured until stage 34 for RT-PCR analysis. **[B]** ImageJ gel densitometry analysis of PCR

Analysis of late pAC reveals that the overall gene expression profile is the same as that seen in control AC/AE. As expected, pAC were negative for expression of the skeletal and neural markers, *MLC1* and *Ntub* respectively. As in the case of the cardiac markers, 2 h of contact time with the AE is sufficient to induce in the pAC all markers observed in the standard AC/AE, with the exception of *For1*. Some markers, namely *Edd, globin,* and *MSR* are even expressed after 1 h of contact. When quantitatively analysed, all markers are also induced to very similar levels observed in control AC/AE (figure 4.5). The lack of *For1* expression itself, either indicates that 2 h is insufficient time to induce liver in the AC and maintained involvement of the AE is required, or more likely that the liver tissue is actually induced in normal AC/AE in the AE itself opposed to the AC. This would support the earlier findings in which terminal liver markers (namely LFABP) were induced due to regional specification of the AE (figure 4.3). With these exceptions however, in this model system it would appear that 2 h of interaction between the inducer (AE) and the responder (AC) is sufficient to result in the induction of a range of cardiovascular cell types.

Using the AC/AE conjugation system, it has been shown that the AE induces robust expression of cardiac markers with only limited other cell fates. Previously, some models of cardiac induction have shown expression of cardiac tissue in response to a more generalised induction of mesoderm (Ariizumi *et al.*, 2003; Logan and Mohun, 1993). It has already been shown that in AC/AE this is not so, at least to the extent of expression of terminal differentiation markers (figure 4.1). It was however, unknown whether this was the case at the time specification. The pAC model permitted investigation of genes expressed immediately upon specification, in isolation away from AE (in the case of whole AC/AE), where endogenous genes expressed would have led to inconclusive results (e.g. induction of *Hex*).

It was therefore next addressed whether activation of these specifically restricted cellular fates observed later in development occurred relatively directly, or was perhaps accompanied by activation of known early targets of mesendoderm induction. Explants were conjugated to the AE and using the peeled AC assay, peeled after 1 or 2 hours of exposure to AE. The AE-free caps were then collected a total of 2.5 h after initiation of contact, and analysed by RT-PCR for expression of known mesendodermal genes (figure 4.12).

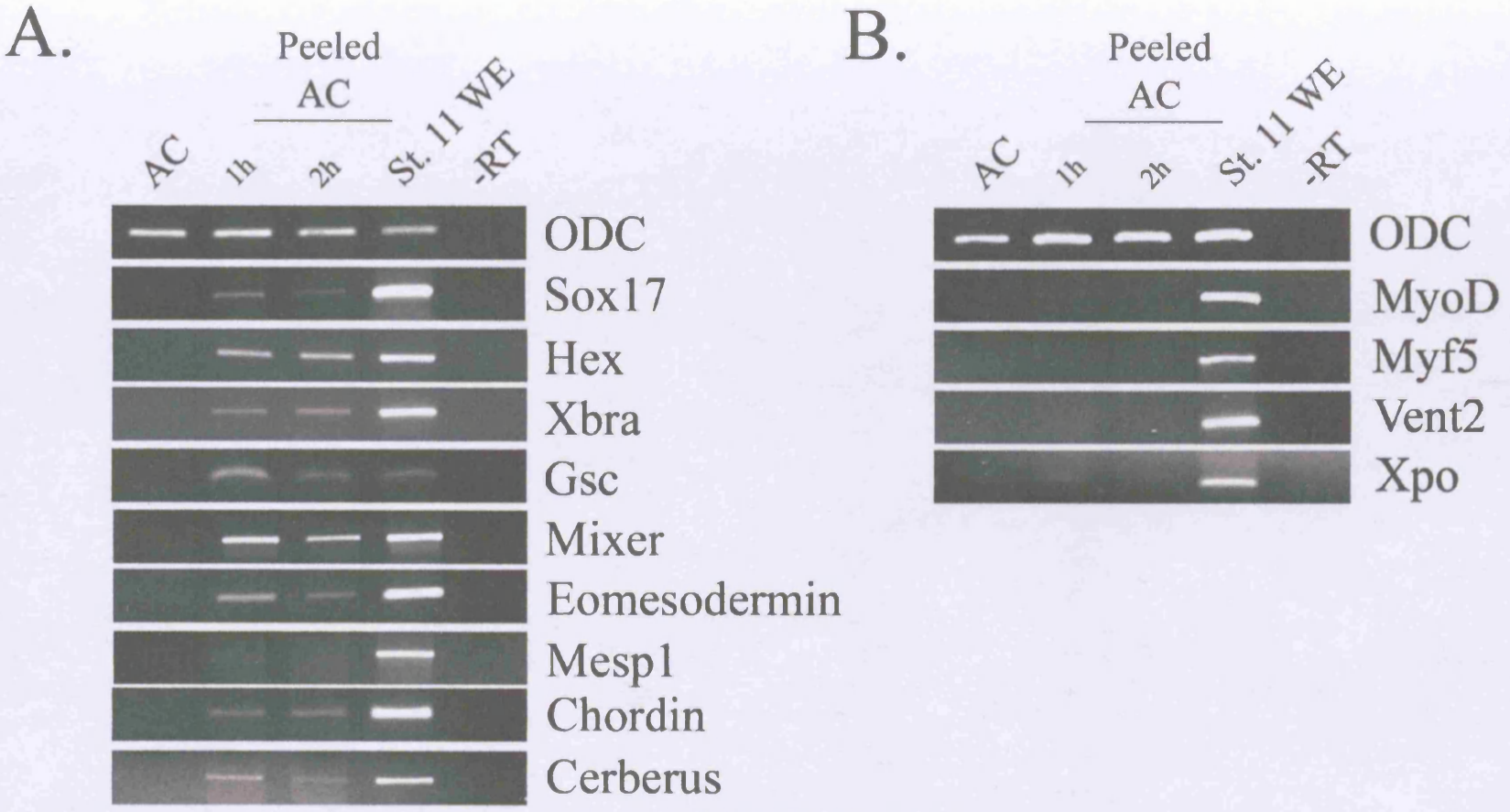


Figure 4.12 – The anterior endoderm induces a range of anterior mesendodermal target genes

Stage 10.25 anterior endoderm (AE) was conjugated to stage 8.5 Animal Cap (AC), and peeled away after 1 or 2 h. Samples were then collected a total of 2.5 h after initial contact for RT-PCR analysis. **[A]** When analysed for a panel of early mesendodermal genes, markers of endoderm (*Sox17, Hex, Cerberus, Mixer*), Organiser (*Goosecoid [Gsc], Chordin*) and mesoderm (*Xbra, Eomesodermin*) are expressed after 1 hour contact with AE. However *Mesp1*, a marker of migrating cardiac precursors, is not induced. **[B]** Analysis for the myogenic regulatory factors *MyoD* and *Myf5*, as well as posterior mesoderm markers *Xpo* and *Vent2*, are not induced by AE.

Analysis of a panel of mesendodermal genes revealed that markers of mesoderm (*Xbra, Eomesodermin*), endoderm (*Sox17, Hex, Mixer, Cerberus*) and the Organiser (*Goosecoid, Chordin*) were all induced in pAC (figure 4.12a). *Eomesodermin* (*Eomes*) is a T-box transcription factor expressed in an equatorial band of all mesodermal cells of the gastrula, in a gradient of increasing concentration from ventral to dorsal. Its expression begins at stage 8 and peaks at early gastrulation before it rapidly declines, at least 2 h earlier than the pan-mesodermal marker *Xbra* (Ryan *et al.*, 1996). Identified in a subtractive PCR screen, *Sox17* (α and β isoforms) were isolated as endodermal enriched SOX mRNAs related to murine *Sox17* (Hudson *et al.*, 1997). Both isoforms are pan-endodermal markers at gastrulation, expressed in the entire endoderm. *Sox17α* expression is maintained in the endoderm until its expression becomes more restricted to posterior endoderm at stage 35, whereas the β isoform is undetectable at these stages. Capable of inducing a range of endodermal markers in AC, it is a key gene in endoderm formation with loss of *Sox17* resulting in loss of endoderm formation (Hudson *et al.*, 1997). Similarly, *Mix-like endodermal regulator* (*Mixer*) homeobox gene was identified from an overexpression screen in which it was specifically shown to induce only endodermal cell-types in AC (Henry and Melton, 1998). Expressed only transiently during gastrulation, *Mixer* is also pan-endodermally expressed with enrichment in the mesendodermal boundary. Knock-down of *Mixer* function severely effects endoderm development and it is required to maintain *Sox17* expression in the embryo. The only exception that was not expressed in pAC was the bHLH transcription factor *Mesp1*, one of the earliest markers of migrating cardiac precursors known to be important in heart development (Saga *et al.*, 2000) which was not induced.

In addition, AC exposed to AE did not express the myogenic regulatory factors *Myogenic factor 5* (*Myf5*; Hopwood *et al.*, 1991) and *Myogenic Determination* (*MyoD*; Hopwood *et al.*, 1989). These bHLH transcription factors have key roles in the establishment of the skeletal muscle cell lineage and the differentiation of myogenic cells, and are known to activate contractile and other myogenic genes (reviewed by Berkes and Tapscott, 2005). As already shown when analysed at stage 34, AC/AE do not show expression of the skeletal tissue (by expression of *MLC1* or staining with 12/101; figure 4.1 and 4.2 respectively). The apparent lack of expression of muscle determinants therefore demonstrates that myogenic differentiation is not initiated nor specified.

Furthermore, posterior markers *Xenopus-posterior* (Xpo; Sato and Sargent, 1991) and *Xenopus Ventral 2* (XVent2; Onichtchouk *et al.*, 1996) were not induced in cells of the pAC (figure 4.11b). *XVent2* (along with *XVent1*) are a class of homeobox genes expressed in the marginal zone of the early gastrula excluding the Organiser, and function downstream of BMP4 to ventralise dorsal mesoderm by antagonising dorsal influences of the Organiser. It is important in specification of ventral mesoderm (Onichtchouk *et al.*, 1996). Similarly, *Xpo* expression begins at MBT but rapidly increases during gastrulation in the posterior mesoderm and ectoderm. Treatment of AC with Activin induces its expression, and it is thought to be important during formation of ventral mesoderm (Sato and Sargent, 1991). The lack of expression of markers of posterior mesoderm shows that the inducing signal(s) from the AE is selective for formation anterior mesendodermal fates, consistent with the *Hex* expressing domain of the AE conferring an anterior signalling centre (figure 4.7; Brickman et al., 2000; Jones et al., 1999).

4.2.3.2 Cardiac fate is likely to be induced directly in conjugates

Analysis of the early genes expressed in cells of the pAC as a result of conjugation to the AE, show both endodermal and mesodermal markers are induced (figure 4.12). Of particular interest was the induction of endodermal markers *Sox17, Hex,* and *Mixer.* Expression of these endodermal determinants suggested endodermal tissue may also have been specified in the AC, supported by the expression of the late pan-endodermal gene *Endodermin* (Sasai *et al.*, 1996). This gene has previously been shown to be induced by *Mixer* and *Sox17* (Henry and Melton, 1998; Hudson *et al.*, 1997, respectively). It was unclear whether the AE induces cardiomyocytes directly or indirectly, by first inducing endodermal tissue in the responder. This therefore raised the possibility that the induced endoderm might influence cardiogenesis.

To address this issue AC were prepared expressing the *Sox17β Engrailed Repressor* fusion protein (Sox17β::EnR; Hudson *et al.*, 1997). Formation of mutant dominant negatives is a frequently employed technique to block gene function. One method is to express mutant versions of heterodimer partners of cell surface receptors such as that of an FGF receptor (Amaya *et al.*, 1991). Alternatively, fusion to the powerful repression domain of *Drosophila* Engrailed protein has been shown to effectively block function of activating transcription factors. *Engrailed* is a homeodomain required for proper segmentation in the fly, and is known to block the activity of a variety of transcription factors (reviewed by Vickers and Sharrocks, 2002). It has been used effectively in *Xenopus* to block the function of a variety of genes, such as *Mixer* (Henry and Melton, 1998), *Xbra* (Conlon *et al.*, 1996), and *Eomes (Ryan et al., 1996). Sox17β::EnR* is a fusion of the Engrailed Repressor domain to the N-terminal of the *Sox17β,* and has been shown to block the function of both *Xenopus Sox17* isoforms. Injection of *Sox17β::EnR;* (1) blocks activin-mediated induction of endodermal, but not mesodermal genes in AC experiments, (2) blocks expression of endodermal genes in vegetal pole explants, causing them to form mesoderm, and (3) affects endoderm formation in whole embryos causing defects in gut formation. The dominant inhibitory construct is therefore a specific inhibitor of *Sox17* function (Hudson *et al.*, 1997). Furthermore, *Sox17β::EnR* has been shown to block both *Sox17* and *Mixer* function in *Xenopus* (Henry and Melton, 1998).

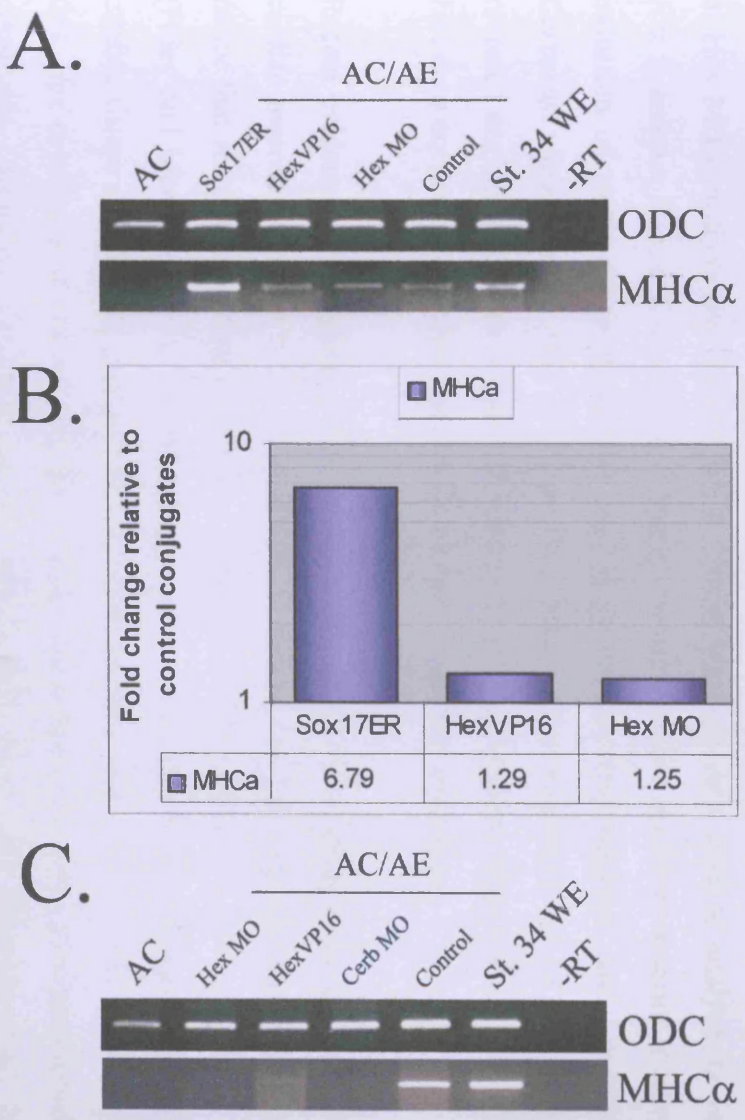


Figure 4.13 – Sox17- and Hex-dependent endoderm in responder is not required for cardiogenesis in AC/AE model

[A] Animal caps (AC) from embryos injected with Sox17ER, Hex-VP16, or Hex MO were conjugated with anterior endoderm (AC/AE) and analyzed for *MHCα* expression when sibling control embryos reached stage 34. **[B]** Quantification of a gel in A. **[C]** AE injected with HexVP16, Hex MO or Cerberus MO (20 ng) was conjugated to AC. Blocking *Hex* or *Cerberus* in the AE blocks cardiac differentiation **[D]** Stage 34 phenotypic controls of *Sox17ER* and *Hex VP16* constructs. Schematic in the lower-right corner indicates the stage and location of injection; 250pg *Sox17ER* in all 4 blastomeres caused loss of posterior endoderm, 500 pg *Hex VP16* injected in dorsal blastomeres of 4-cell embryo caused posteriorisation, 20 ng of Hex MO result in all blastomeres of the 4-cell embryo resulted in anterior truncations and patterning defects.

Conjugates in which AC explants were expressing dominant-negative Sox17β protein expressed higher level of cardiac markers compared to control (figure 4.13a). This suggests that in AC/AE explants the AE induces cardiac fate independently of the endoderm induced in the AC. Furthermore, *Sox17*-dependent endoderm appears to oppose cardiogenesis with a greater level of cardiac marker expression observed upon inhibition of *Sox17* function. A similar result was previously described in animal caps in which mesoderm and endoderm were induced by GATA4 (Latinkic *et al.*, 2003).

It was shown that pAC express the homeobox gene *Hex*, known to mark the AE at early gastrula stages (Newman *et al.*, 1997). In addition, *Hex* confers an anterior signalling centre (Jones *et al.*, 1999; Zorn *et al.*, 1999) and promotes cell non-autonomous anteriorisaton of the embryo through suppression of *Gsc* and *Chordin*, genes of the Organiser (Brickman *et al.*, 2000). It has also been shown that the cardiac-inducing signal of the AE in AC/AE correlates with the *Hex* expressing domain (figure 4.7). Work by Schneider and Mercola (2001) showed that induction of cardiac tissue in the VMZ was mediated by induction of *Hex,* with its transcriptional repressive function important (Foley and Mercola, 2005).

It was therefore investigated whether induction of *Hex* was required for cardiogenesis in the AC/AE cardiac model by using AC expressing *Hex VP16* mRNA (Brickman *et al.*, 2000) or Hex MO (Smithers and Jones, 2002). These reagents were used to analyse the role of *Hex* in anterior fate, and it was found that its overexpression produces anterior truncations, induction of trunk dorsal mesoderm and early mesodermal markers, and inhibition of *Cerberus*. Injection of *Hex VP16* or Hex MO in the responder of AC/AE however, revealed negligible effects of terminal cardiac marker expression (figure 4.13a). Therefore, *Hex* does not mediate cardiogenesis downstream of cardiac induction.

Recent evidence has suggested that the requirement for Wnt antagonism in induction of cardiac mesoderm is due to its requirement to pattern the AE and regulate secretion of a factor that results in cardiac induction. This was claimed to be via *Hex* expression itself (Foley and Mercola, 2005). Furthermore, *Cerberus* has also been implicated in inducing cardiac tissue in a distinct pathway downstream of Xnrs (Foley *et al.*, 2007). To address this, *Hex* expression in the AE was blocked by overexpression of the aforementioned *Hex* constructs. Similarly, *Cerberus* was blocked by MO expression (Kuroda *et al.*, 2004).

Embryos were injected at the 2-cell stage in ventral regions and AE extirpated at stage 10 for conjugation. Ability to induce cardiac tissue was determined by expression of terminal cardiac markers, and compared to uninjected control AC/AE (figure 4.13c). As expected, overexpression of these constructs blocked cardiac differentiation in AC/AE.

4.3 Summary

4.3.1 Anterior endoderm produces cardiovascular specific signal

In this chapter it was shown that in addition to cardiac tissue, AE induced markers of other cell types involved in cardiovascular development; endothelium, macrophages, smooth muscle, and blood. It is important to note however, that the AE (with the exception of *Hex*) showed no expression of these markers, and thus their expression in AC/AE was the result of conjugation. Importantly, AC/AE explants were free from skeletal muscle (and neural tissue) demonstrating that AE was not inducing cardiac tissue as a part of general mesoderm induction, but was acting relatively specifically to induce a finite selection of different but related, cell fates. Interestingly, AC/AE showed expression of *For1,* indicative of liver fate.

4.3.2 Cardiac inducing activity correlates with Hex expression

Upon investigation of the signalling source, it was revealed that inducing capacity was localised to the most anterior regions of the AE. Dividing the AE into more posterior and anterior regions led to a greater ability to induce cardiac fate in the anterior AE, opposed to a marked decrease in conjugates of the posterior AE. Furthermore, this appeared to correlate closely with an increase in *Hex* expression in anterior conjugates, a marker known to confer anterior identity in the early *Xenopus* embryo. Such a finding was also apparent when a comparison was made between the cardiac inducing capacity of the AE and vegetal conjugates of blastula endoderm. In conjugates of stage 8 vegetal poles and those aged until stage 10 was cardiac tissue never observed, but robust expression of markers of neural and skeletal muscle cell fates was apparent. In addition, neither of these inducing sources

showed *Hex* expression in contrast to that of the AE. These results demonstrate that the two inducing tissues produce distinct signals, which is the result of some fundamental difference between the inducing capacity of the vegetal pole and AE. Therefore, the vegetal pole likely requires further interactions *in situ* to be specified as a cardiac inducing source.

4.3.3 Cardiac inducing signal is active during early gastrula stages

The ability to readily form cardiac tissue upon conjugation permitted signalling and inducing sources to be manipulated to further characterise cardiac specification. By conjugating AC to increasingly older samples of AE, it was found that cardiac-inducing capacity was severely reduced by mid gastrulation; cardiac induction was completely absent from conjugates of late gastrula AE. Similarly, the AC responder was only competent to respond to cardiac induction during late-blastula to early-gastrula stages, as cardiac marker expression was barely detectable by stage 13.

4.3.4 Two hours of contact with the AE is sufficient to induce cardiogenesis

Greater refinement of the timing of cardiac specification was revealed by peeling AC from the inducer after a defined period of time. It was shown that 2 hours of contact was sufficient to induce cardiac differentiation markers to the same level induced in control conjugates. Furthermore, using transfilter studies it was shown that induction of cardiac fate was the result of a diffusible factor as the filter did not abrogate cardiac marker expression.

Characterisation of the cellular fates induced in AC peeled from the AE revealed most cell fates induced in control conjugates resulted after 2 hours of contact. Notably, *Forl* was not induced in the pAC after 1 or 2 hours, which may be the result of reciprocal signalling from the cardiac mesoderm in the AC to induce liver in cells of the AE. Upon analysis of early markers induced in the peeled AC, it was found that a range of endodermal and mesodermal markers were induced. Further to the specificity of the AE in cardiogenesis,

skeletal muscle fate was never initiated as the master regulators *MyoD* and *Myf5* were never induced. Also, the AE was selective for fates of anterior mesoderm as posterior markers were not expressed.

Expression of markers of the endoderm in the AC raised the possibility that cardiac mesoderm was induced indirectly. This was found however, not to be the case as *Hex-*, *Sox-*, and *Mixer* dependent endoderm was not required in the AC for cardiac marker expression as blocking their expression had no effect. This therefore implies that in AC/AE cardiac tissue is induced directly, and the model provided the basis for investigation into the signalling events responsible for specification of the cardiac program.

CHAPTER 5 – INVESTIGATING THE SIGNALLING REQUIREMENTS FOR CARDIAC SPECIFICATION

5.0 INVESTIGATING THE SIGNALLING REQUIREMENTS FOR CARDIAC SPECIFICATION

5.1 Introduction

Previous work has proposed several signalling pathways in specification of cardiac fate, but questions of specificity and a direct involvement of the named players were unclear. Given the experimental accessibility and directness of the AC/AE assay, it provided the opportunity to examine the thus far unresolved events of cardiac specification.

To achieve this, the signalling capacity of the responding AC was modified by manipulating the signalling pathways thought to be involved in cardiac induction. Modifications of the inducing source were avoided to prevent interference with germ layer specification, as doing so may have indirectly affected cardiogenesis by altering the inductive capacity of the AE. Evidence for this has already been shown (figure 4.13c). As previously, the involvement of different signalling pathways was scored by analysing expression of cardiomyocyte-specific differentiation markers at tadpole stages when they are robustly expressed. This was however prior to the advanced stages of heart growth and morphogenesis, when different signalling molecules may have distinct roles from those of specification and differentiation. Analysis of differentiation markers was the only possible readout for study into to early specification events given the lack of adequate early cardiac-specific markers.

5.1.1 Chapter Aims

Detailed analysis into signalling requirements for cardiac specification was made using a range of activators/inhibitors of implicated signalling pathways. Effects on specification were determined by changes in expression of terminal differentiation markers. It was therefore aimed to:

- Investigate the requirements for signalling pathways implicated in cardiac specification in the AC/AE model; FGF, BMP, Nodal, Wnt/β-catenin

- Determine the time of action of required pathways to accurately describe the sequence of events required for specification of cardiac precursors

5.2 Results and Discussion

5.2.1 FGF signalling is required for cardiac induction

5.2.1.1 Inhibition of FGF signalling in conjugates blocks cardiac specification

To investigate FGF signalling in conjugates, the ability of the AC to respond to FGF ligands was perturbed using a combination of different inhibitors that blocked the pathway at different cellular levels; a dominant negative form of the FGFR1 (*ΔFGFR1*; Amaya *et al.*, 1991), a chemical inhibitor of FGFR1 signalling (SU5402; Mohammadi *et al.*, 1997), and a chemical inhibitor of MAPK signalling (U0126; Favata *et al.*, 1998). *ΔFGFR1* is a mutated form of the wildtype FGFR1, consisting of intact extracellular and transmembrane domain, but with no intracellular domain thus preventing signal transduction. Expression of the receptor has been shown to prevent AC responsiveness to FGF mesoderm induction, and overexpression in the embryo results in extreme trunk defects and deficiencies in lateral and posterior mesoderm. Embryos undergo incomplete gastrulation with failure of blastopore lip closure, hence preventing formation of an intact dorsal axis (Amaya *et al.*, 1991). SU5402 is a tyrosine kinase domain inhibitor of FGFR1, inhibiting the auto-phosphorylation that occurs upon binding of FGF ligand to receptor (Bottcher and Niehrs, 2005). Composed of an oxindole core it occupies the binding site of ATP, resulting in a conformational change preventing nucleotide binding, and has been shown to prevent downstream ERK phosphorylation (Mohammadi *et al.*, 1997). It has been used extensively to inhibit FGF signalling (Alsan and Schultheiss, 2002; de Pater *et al.*, 2009; Marques *et al.*, 2008). Treatment of *Xenopus* embryos with SU5402 causes incomplete blastopore closure, short embryonic trunk, indistinguishable anterior-posterior structures; a phenotype closely resembling *ΔFGFR1* although apparently more severe (Chung *et al.*, 2004). Lastly, U0126 is a non-competitive selective inhibitor of Mitogen Activated Protein Kinase Kinase (MAPKK). This prevents subsequent phosphorylation of extracellular signal-regulated kinases (ERK; Davies *et al.*, 2000; Favata *et al.*, 1998; Ilagan *et al.*), one of the intracellular pathways responsible for transduction of FGF signalling (Bottcher and Niehrs, 2005).

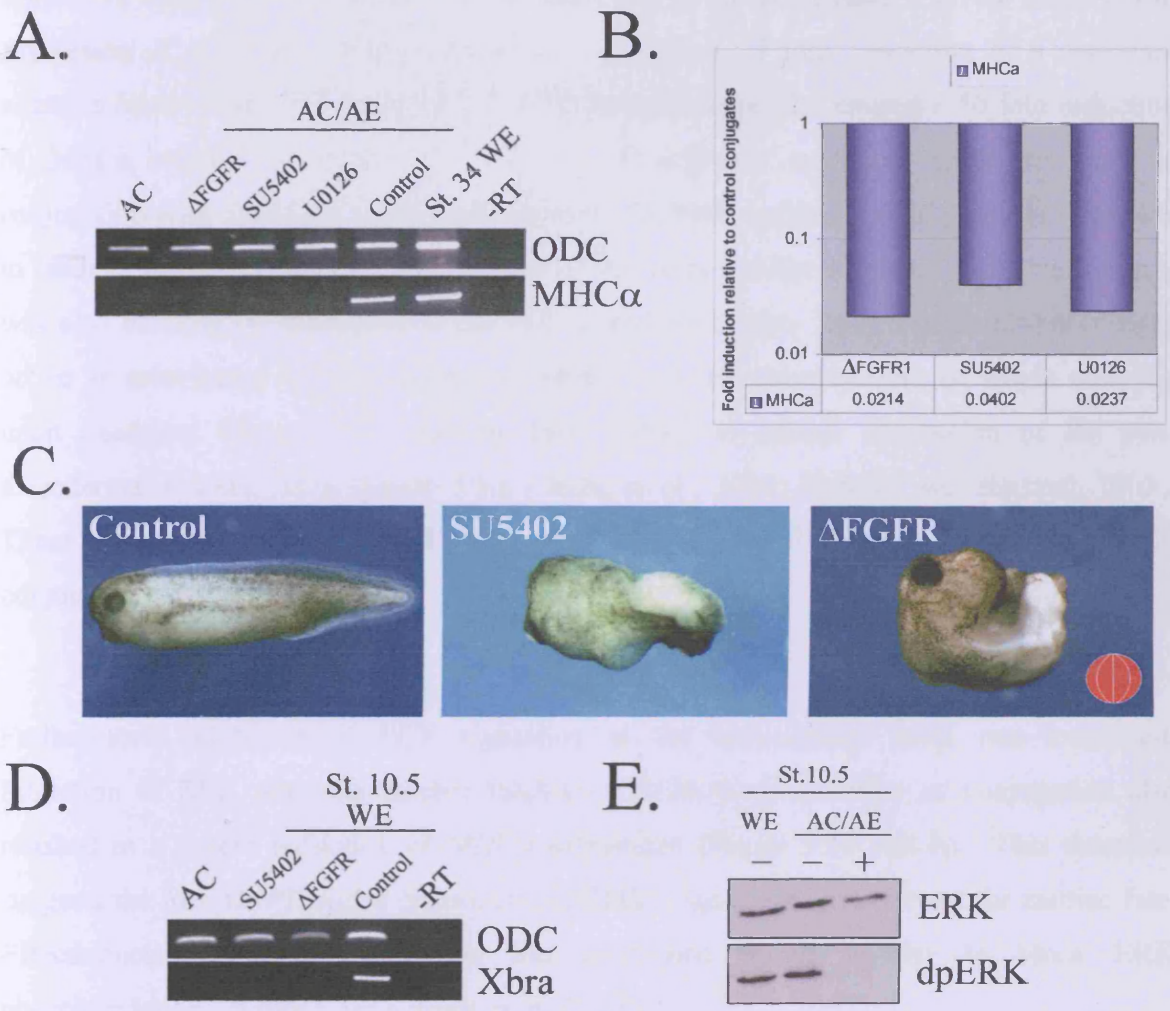


Figure 5.1 – FGF signalling is required for expression of terminal cardiac differentiation markers

[A] AC/AE conjugates were made with animal cap (AC); expressing ΔFGFR (1 ng), uninjected and continuously incubated with SU5402 or U0126 from the time of conjugation, or left untreated (Control). MLC2 expression was analyzed when control siblings (WE) reached stage 34. **[B]** ImageJ analysis of PCR data. **[C]** Phenotypic analysis of embryos injected with ΔFGFR or those treated continuously with SU5402, showing severe posterior truncations. **[D]** Loss of *Xbra* expression in stage 10 whole embryo treated with SU5402 or universally injected with ΔFGFR, compared to control embryos. **[E]** Protein analysis of AC/AE one hour after conjugation (-) shows strong expression of phosphorylated ERK (dpERK) which is abolished upon treatment with U0126 (+) immediately upon conjugation. Total ERK was used as a loading control.

Figure 5.1 shows that inhibition of FGF signalling in AC/AE causes a severe reduction in expression of terminal cardiac differentiation markers. Firstly, injection of a dominant negative form of the FGF receptor (*ΔFGFR*) into the responder caused a 50-fold reduction of *MHCα* expression (figure5.1a and b). Continuous treatment from the time of conjugation with the FGFR1 chemical inhibitor, SU5402, caused a similar severe reduction in cardiac marker expression. Expression of the early cardiac markers, *Nkx2.5* and *Tbx5,* was also blocked by inhibition of the FGF pathways (figure 5.5c). Both inhibitors were active as ascertained by their ability to inhibit posterior development of whole embryos upon treatment (figure 5.1c) and by their ability to inhibit expression of the pan-mesodermal marker, *Xbra* (figure 5.1d; Chung et al., 2004; Fletcher and Harland, 2008). These results indicate that FGF signalling is essential for cardiogenesis in AC/AE conjugates.

Furthermore, inhibition of FGF signalling at the intracellular level was examined. Inhibition of ERK with the soluble inhibitor U0126 from the time of conjugation also resulted in a severe reduction of *MHCα* expression (figure 5.1a and b). This therefore suggests the *Ras/MAPK* signal transduction of FGF signalling is important for cardiac fate. Effectiveness of U0126 treatment was confirmed by its ability to block ERK phosphorylation (figure 5.1e; Kuroda et al., 2005).

From these results it was unclear to what extent FGF signalling was required for cardiac development in AC/AE and whether it was needed to induce cardiac fate. There is evidence to suggest that FGF signalling merely acts as a competence factor in synergy with BMP to promote cardiac development (Ladd *et al.*, 1998; Lough *et al.*, 1996). Furthermore, FGF signalling in early embryonic development is known to be required to maintain mesodermal precursors induced by TGFβ (Cornell and Kimelman, 1994; Cornell *et al.*, 1995; Schulte-Merker and Smith, 1995). From RT-PCR analysis it could not be concluded whether there was; (a) complete loss of cardiac marker expression in a proportion of conjugates, or (b) a reduction in cardiac marker levels in all conjugates. To resolve this, conjugates were made of AE with AC overexpressing *ΔFGFR1* and analysed by WMISH to study cardiac gene expression (figure 5.2).

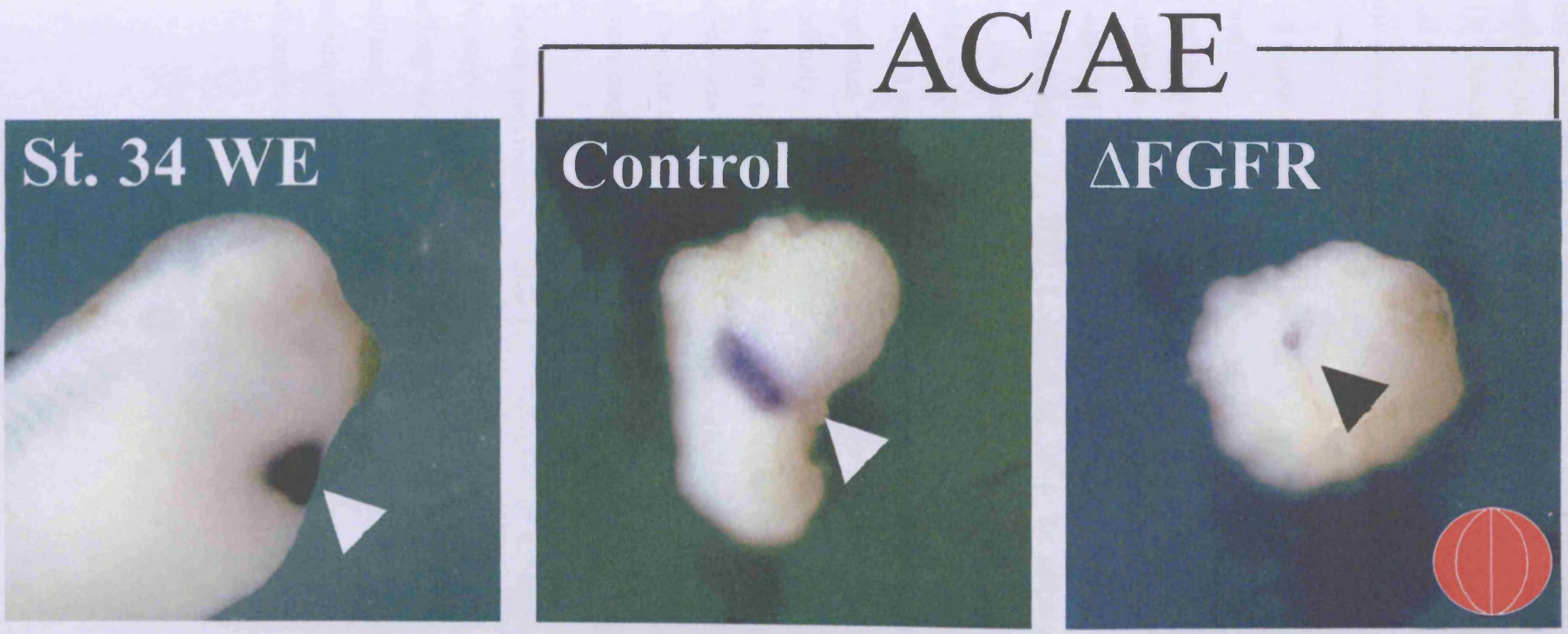


Figure 5.2 – FGF signalling in the AC is essential for efficient cardiac induction in AC/AE

WMISH of stage 34 conjugates (AC/AE) reveals ΔFGFR greatly reduces **CTnI** expression. Control explants show a larger single focus of staining (white arrowhead; 83% (n=11)), whereas expression of ΔFGFR1 in animal caps leads to a greatly reduced expression (black arrowhead) in 89% (n=19) explants. Schematic in the lower-right corner indicates the stage and location of injection. Expression of CTnI in the endogenous heart of stage 34 whole embryo (WE) is shown.

As previously described, figure 5.2 shows AC/AE exhibit robust expression of cardiac differentiation markers (in this case CTnI) in a single focus adjacent to the inducer (83%; n=11). However, uniform injection of the dominant negative receptor in the responder caused a severe reduction in CTnI expression, and the extent of cardiac positive cells was greatly reduced.

5.2.1.2 Cardiac induction in AC/AE requires FGF signalling in the first hour of contact

The requirement for FGF signalling in cardiogenesis supports previous evidence suggested in vertebrates (Alsan and Schultheiss, 2002; Reifers *et al.*, 2000). However, previous work has suggested a more apparent later role in maintenance of cardiac precursors (Ladd *et al.*, 1998; Langdon *et al.*, 2007; Lough *et al.*, 1996; Schultheiss *et al.*, 1997). To address these issues, the timing requirements for FGF signalling were explored using various treatments with the soluble inhibitor, SU5402. Conjugates of uninjected control tissues were made as normal, but exposed to different time-window treatments to determine when FGF signalling is important for cardiac development. Initially, broad treatment windows were used; immediately after conjugation during gastrulation (responder stage 9 to 12), and after gastrulation (responder stage 12 to 34; figure 5.3a). The rationale for such treatment windows was to determine the sequence of events involved in cardiac specification, as it was possible that the apparent down-regulation of cardiac markers upon treatment of FGF inhibitors could be due to a later involvement, after the initial specification events.

As shown previously, continuous treatment of AC/AE with SU5402 from the time of conjugation severely affected cardiac marker expression. Furthermore, inhibition of FGF signalling was found to reduce *MLC2* expression to the same extent when treated during gastrulation. Treatment after gastrulation however, had no effect on cardiac gene expression (figure 5.3b). This suggested a requirement for FGF signalling early in development during the time of cardiac specification.

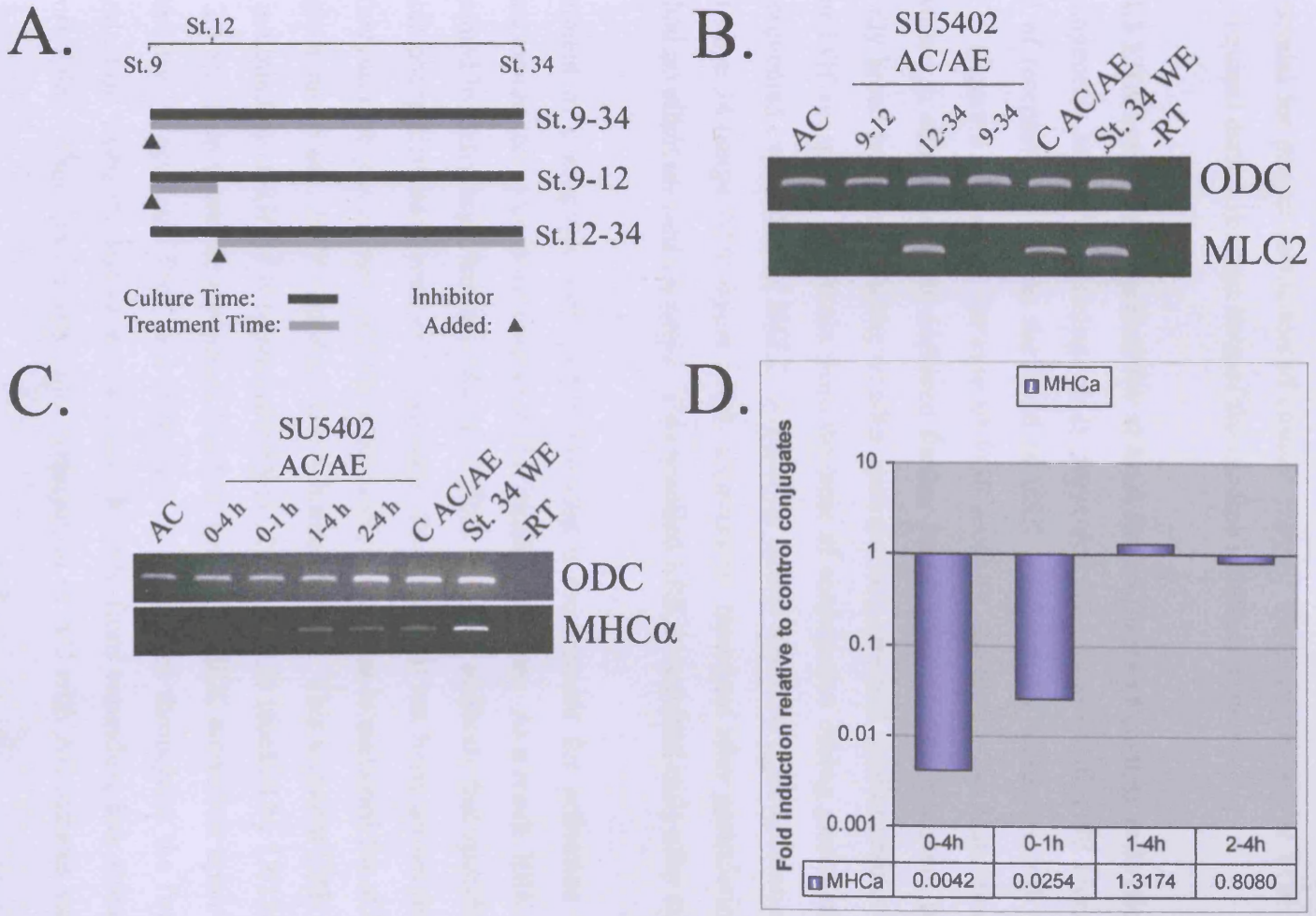


Figure 5.3 – FGF signalling is required during the first hour for robust expression of cardiac markers

[A] Schematic of timed inhibitor treatments of conjugates (AC/AE). **[B]** RT-PCR analysis of stage 34 AC/AE treated with SU5402 during the treatment windows; stage 9 to stage 12 (9-12), stage 12 to stage 34 (12-34), and throughout cultivation (9-34). Treatment blocked *MLC2* expression in the early, but not later window, compared to control conjugates (C AC/AE). **[C]** Further refinement of inhibitor treatment windows (length of incubation indicated), shows FGF signalling is required in the first 4 h after conjugation (0-4 h), with an absolute requirement in the first hour (0-1 h), but not after. Corresponding ImageJ analysis of RT-PCR data is shown in **[D]**.

To further dissect out the role of FGF the window of involvement of FGF signalling was analysed with finer resolution, focusing on the first 4 hours of incubation (corresponding approximately to stage 11). Continuous presence of SU5402 during the first 4 hours of conjugation prevented cardiogenesis, as ascertained by a severe reduction in *MHCα* expression (0-4 h, figure 5.3). Similarly, brief treatment of AC/AE during the first hour of conjugation blocked cardiogenesis to the same extent (0-1 h). In contrast, SU5402 treatment after 1 hour of contact had no obvious effects on cardiogenesis. Taken together these results demonstrate that the FGF signalling pathway acting at the level of the receptor is essential for proper formation of cardiac tissue. Furthermore, active FGF signalling is only required during the first hour of the cardiac induction process.

5.2.1.3 ERK activity is required for at least first 4 hours of cardiac induction

Cardiogenesis in AC/AE conjugates is prevented by inhibition of FGF signalling at the level of receptor, but also at the level of ERK as shown by treatment with the inhibitor U0126 (figure 5.1). As in the case of FGF receptor signalling, the requirement for ERK activation in conjugates was explored further by timed inhibition treatment using U0126. Initially broad treatment (during or after gastrulation) revealed similar results to inhibition of the FGF receptor. Treatment from the time of conjugation during gastrulation (stage 9-12) prevented expression of *MLC2,* to a similar extent observed upon continuous treatment until stage 34 (stage 9-34; figure 5.4a). Conversely, treatment after gastrulation (stage 12-34) had no effect on cardiogenesis. This revealed ERK is required early after conjugation.

Treatment of conjugates with U0126 revealed a requirement for activation of ERK for robust expression of terminal cardiac differentiation markers. As a result, ERK activity was examined by detecting phosphorylation of ERK, using an antibody that specifically detects double phosphorylated forms of the protein. Previously, it has been shown that wounding of *Xenopus* embryos causes transient activation of ERK that is sustained for at least an hour (Christen and Slack, 1999; LaBonne and Whitman, 1997). This transient ERK activation is not inhibited by *ΔFGFR1* (Christen and Slack, 1999), but is blocked by U0126 (Kuroda *et al.*, 2005). This therefore prevented accurate timing of ERK activation upon conjugation. Irrespectively, activated ERK was detectable in conjugates throughout the first four hours of induction when the transient activation of ERK from wounding has already been lost (figure 5.4b). This demonstrates that conjugation of AC with AE induces sustained ERK

activity. Phosphorylated ERK was however not detectable by stage 14, supporting the early sensitivity of conjugates to U0126.

When conjugates were subjected to U0126 during this time period (i.e. during the first 4 hours following conjugation) phosphorylation of ERK was prevented (figure 5.4e) and substantially reduced cardiogenesis (0-4 h; figure 5.4c). The level of inhibition was similar to that previously shown for SU5402 treatment. Further refinement of this 4 h period into two shorter time-windows (0-2 and 2-4 h) of treatment with U0126 reduced *MLC2* expression by approximately 2-fold (figure 5.4c). This is in contrast to SU5402, which showed no effects on cardiac marker expression after the initial hour (figure 5.3). Hence, two hours of ERK activity during the first 4 hours of induction is still sufficient for cardiogenesis to some extent. The level of cardiac marker expression however, is only half as efficient as in control AC/AE conjugates (figure 5.4d).

One implication of this result is that ERK activity is required during the entire 4 hour period for full maximal cardiogenesis (relative to control AC/AE explants), after which point it is not required. The extent of cardiac tissue formed however, seems to correlate closely with the level (or duration) or ERK activity to which the responder is exposed to. In contrast, the SU5402 experiments reveal an *absolute* requirement for FGF signalling in the first hour of induction. This difference is also apparent in a comparison of treatment of AC/AE explants with SU5402 during the 1-4 h time window (with no effect on cardiogenesis), with a shorter U0126 treatment (during the 2-4 hour period) leading to two-fold reduction in cardiogenesis. In summary, manipulation of both FGF and ERK signalling pathways suggests that FGF signalling at the level of receptor activation is required for cardiogenesis only during the first hour of induction. Sustained ERK activity is however, necessary throughout the first four hours of induction.

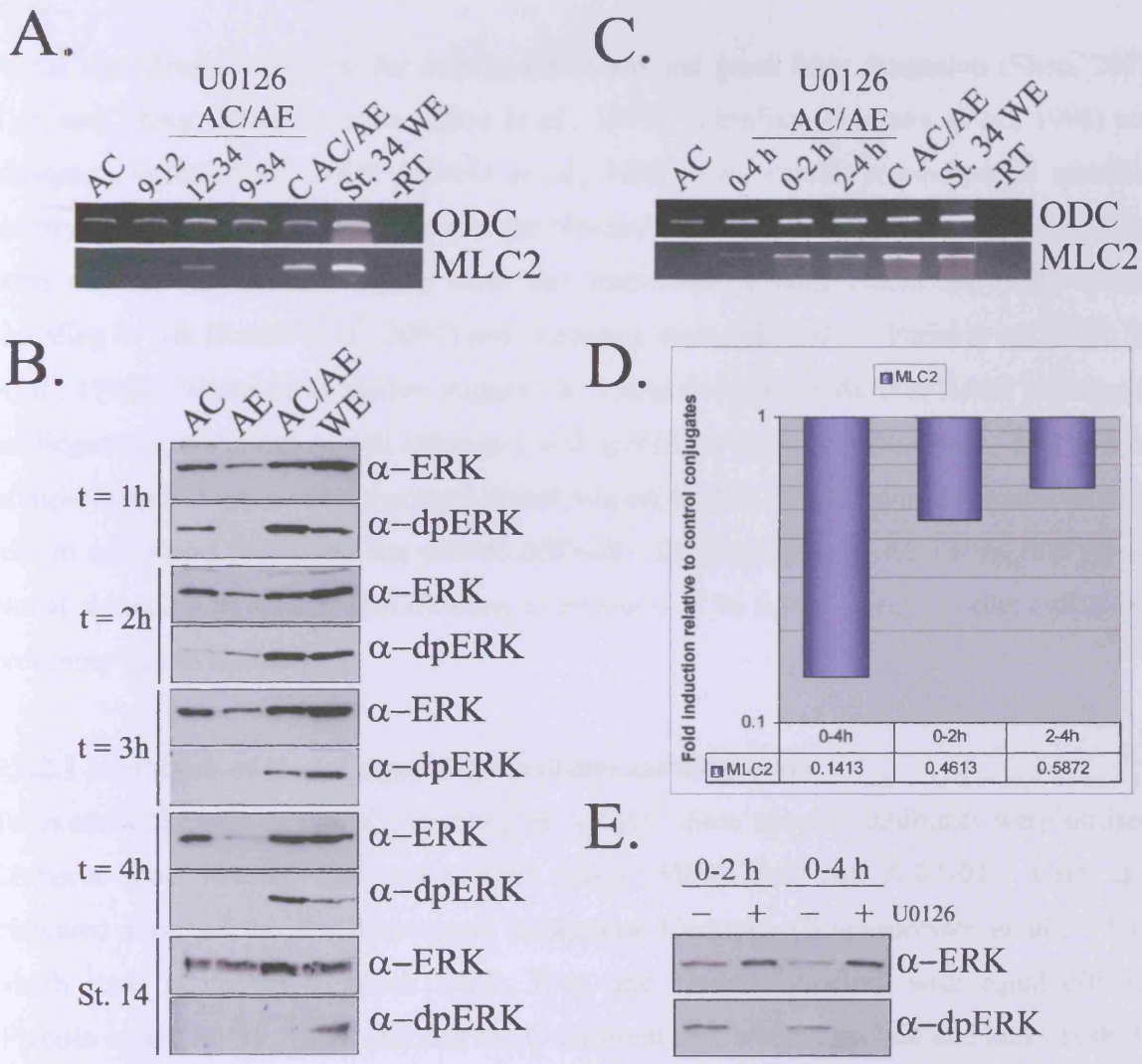


Figure 5.4 – ERK is activated during the first 4 hours and is required for cardiogenesis over the same period

[A] RT-PCR analysis of broad U0126 treatment windows of stage 34 conjugates (AC/AE). ERK is required for cardiac specification during gastrulation (stage 9-12) but not after it (stage 12-34). **[B]** Animal cap (AC), anterior endoderm (AE) or AC/AE explants were collected at indicated times after conjugation and were subjected to Western analysis for double-phosphorylated ERK (dpERK) and total ERK. The latest time-point (St. 14) corresponds to 8-10 hours after conjugation. ERK is activated in AC/AE for at least 4 hours after conjugation. **[C]** Further refinement of ERK requirement. U0126 treatment for the 2 or 4 hours after conjugation reduces *MLC2* expression (0-2 h and 2-4 h), with gel densitometry analysis **[D]** showing approximately 2-fold reduction in levels of cardiac gene expression. Treatment for 4 h reduces expression 10-fold (0-4 h). **[E]** Western control reveals treatment of AC/AE for 2 and 4 h effectively abolishes ERK phosphorylation.

5.2.2 Nodal signalling directly induces cardiac fate

Nodal signalling is essential for axis specification and germ layer formation (Shen, 2007; Tian and Meng, 2006) in mice (Zhou *et al.*, 1993), zebrafish (Feldman *et al.*, 1998) and *Xenopus* (Agius *et al.*, 2000; Piccolo *et al.*, 1999). As cardiac mesoderm is specified shortly afterwards, the precise role that the Nodal/Activin pathway plays in this process has been difficult to define. Much work has implicated a requirement for active Nodal signaling in fish (Reiter *et al.*, 2001) and in murine stem-cell studies (Parisi *et al.*, 2003; Xu *et al.*, 1998). While these studies suggest an instructive role of Activin/Nodal pathway in cardiogenesis, questions of cell autonomy and specificity remain unanswered. Due to roles in mesoderm induction, deciphering a direct role on cardiac specification independent of its role in mesoderm formation has proved difficult. Utilising the AC/AE model, the role of Nodal signalling in cardiac specification in isolation of its involvement in other embryonic processes was evaluated.

5.2.2.1 Inhibition of Nodal signalling abolishes cardiogenesis

To examine the role of Nodal signalling in AC/AE, three specific inhibitors were utilised; Cerberus-Short (CerS), and two soluble drugs, SB-431542 and A-83-01. CerS is a truncated form of the multifunctional antagonist Cerberus (Bouwmeester *et al.*, 1996), which itself is known to block BMP, Wnt, and Nodal signalling with equal efficacy (Piccolo *et al.*, 1999). *CerS* encodes the C-terminal of Cerberus protein and lacks both the BMP and Wnt inhibitory domains, and thus blocks Nodal signalling. Overexpression of *CerS* in the embryo has been shown to block both dorsal and ventral mesoderm formation with failure of gastrulation (Piccolo *et al.*, 1999). It has also been shown to inhibit multiple *Xnrs*, specifically only the mesoderm inducing Nodal proteins, as it does not inhibit Xnr3 (Agius *et al.*, 2000). SB-431542 is a competitive ATP-binding site, kinase inhibitor that prevents phosphorylation of corresponding R-Smads of the appropriate transduction pathway (Callahan *et al.*, 2002). It is a potent and selective inhibitor of Activin and TGFβ signalling via inhibition of ALK4, ALK5, and ALK7, and therefore blocks phosphorylation of Smad2 and 3. The compound however has no effects on BMP, ERK, JNK, or p38 MAPK pathways (Inman *et al.*, 2002). Specifically ALK4 and ALK7 are used by the activin and nodal signalling pathways respectively (Reissmann *et al.*, 2001). It therefore has been shown to specifically block nodal signalling in *vivo* in *Xenopus* and zebrafish

(Hagos and Dougan, 2007; Ho *et al.*, 2006). Treatment of embryos results in a phenotype typical of Nodal knockdown, preventing the expression of downstream targets such as *Goosecoid* and *Brachyury,* but importantly the effects of the compound are reversible (Ho *et al.*, 2006). Similarly, A-83-01 is a more potent inhibitor of ALK4, 5, and 7 shown to specifically block Nodal signalling in cell culture assays (Tojo *et al.*, 2005).

AE was conjugated to AC, injected with *CerS,* or uninjected and treated with the chemical inhibitors from the time of conjugation (figure 5.5a). It was found that all three nodal inhibitory reagents efficiently blocked cardiogenesis in AC/AE explants, with at least a 200-fold reduction in *MHCα* expression. This was a much more significant level of inhibition than that observed upon inhibition of FGF. WMISH analysis of AE conjugated to AC overexpressing *CerS* at stage 34 showed a similar failure of AC/AE to undergo cardiogenesis with no detectable expression of *CTnI* (figure 5.5c). In addition, conjugates treated with A-83-01 were analysed at stage 18 and showed no expression of the cardiac transcriptional regulators *Nkx2.5* and *Tbx5* (figure 5.5c). Positive activity of inhibitors was confirmed by their ability to prevent expression of *Xbra* (figure 5.5d; Ho et al., 2006), and phenotypically via their ability to cause gastrulation arrest (figure 5.5e; Agius et al., 2000; Ho et al., 2006). These results suggest Nodal signalling in the AC/AE model is essential for formation of cardiac tissue.

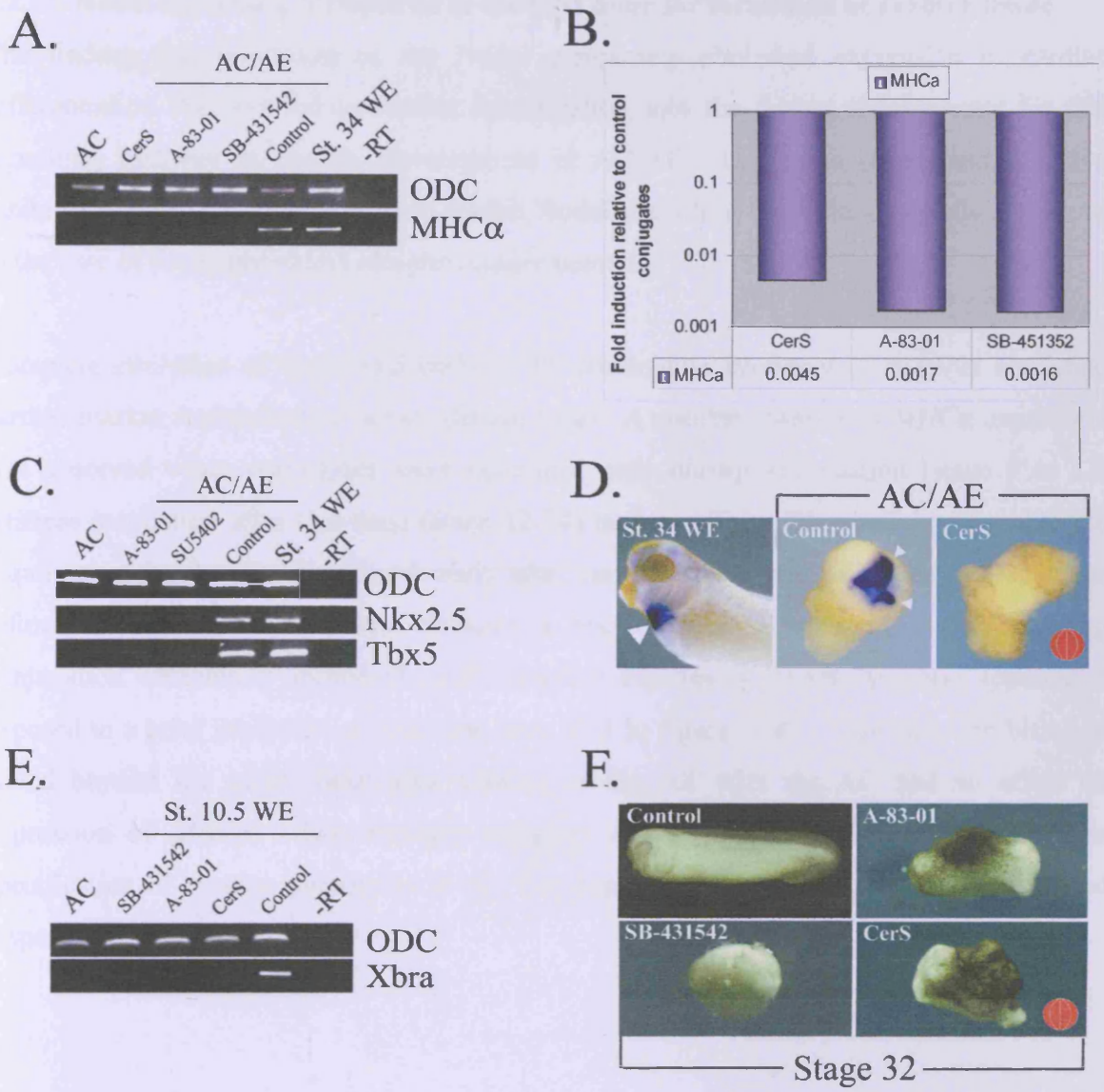


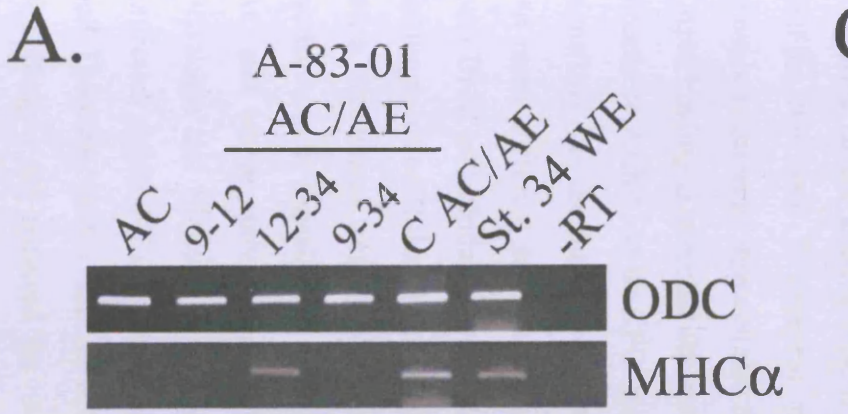
Figure 5.5 – Blocking Nodal signalling abolishes cardiac marker expression

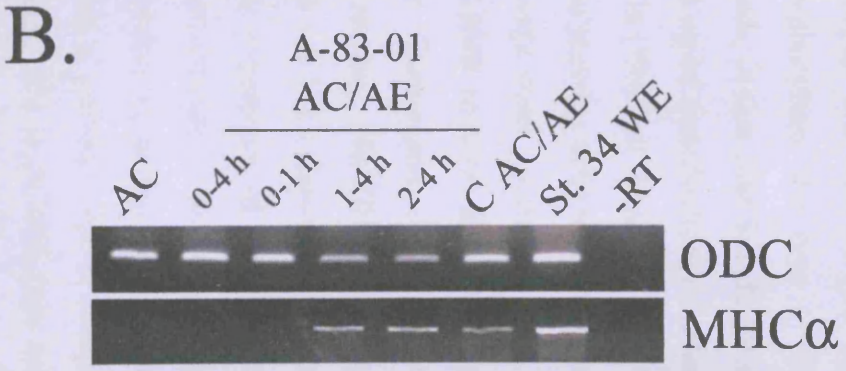
[A] Cardiac marker analysis of stage 34 conjugates (AC/AE) in which nodal signalling was inhibited. AC/AE conjugates were made with animal cap (AC); expressing CerS (1 ng), uninjected but continuously incubated with SB-431542 or A-83-01 from the time of conjugation, or left untreated (Control). All treatments effectively block cardiogenesis when compared to control, with gel densitometry readings shown in **[B]**. **[C]** Expression of the early cardiac markers *Nkx2.5* and *Tbx5* is blocked by inhibition of FGF and Nodal signalling. **[D]** WMISH analysis revealed CerS injection in AC prevents expression of **CTnI** (white arrowhead) in AC/AE explants (0/15 of CerS explants express CTnI, in contrast to 12/15 control explants). Schematic in the lower-right corner indicates the stage and location of injection. **[E]** Control for nodal inhibition. RT-PCR analysis of A-83-01 or SB-431542 treated whole embryo (WE), or those injected with CerS, revealed loss of *Xbra* expression when compared to untreated sibling embryos. **[F]** Phenotypic analysis confirms complete nodal inhibition, revealing defective gastrulation upon all treatments

5.2.2.2 Nodal signalling is required in the first hour for formation of cardiac tissue

The finding that inhibition of the Nodal completely abolished expression of cardiac differentiation markers led to further investigation into the timing requirements for this signalling pathway in cardiac development in AC/AE. Using the potent and selective inhibitor A-83-01, it was possible to inhibit Nodal signalling for defined periods of time as in the case of the experiments into the requirements for FGF.

Complete inhibition of the Nodal pathway by continuous treatment in A-83-01 abolished cardiac marker expression as before (figure 5.6a). A similar absence of *MHCα* expression was observed when conjugates were incubated only during gastrulation (stage 9 to 12) whereas incubation after this time (stage 12-34) had no effect. This indicated an absolute requirement for the Nodal pathway early after conjugation of the AC to the AE. Further refinement of this time-window revealed a brief 4 hour treatment (0-4 h) following conjugation completely abolished cardiac marker expression, which was also apparent if exposed to a brief inhibition of only one hour (0-1 h; figure 5.6b). However, inhibition of Nodal beyond the initial hour after contact of the AE with the AC had no effect on expression of *MHCα*. This strongly suggests active Nodal signalling is involved in specification of cardiac precursors in the first hour upon conjugation of the inducer and responder.





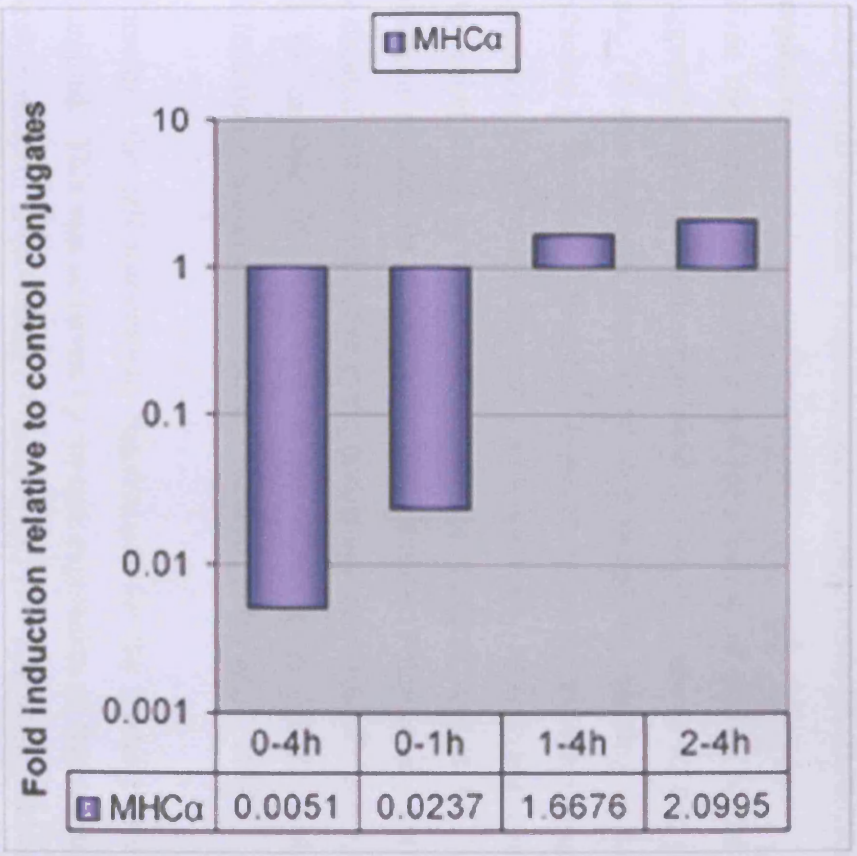


Figure 5.6 – Nodal signalling is necessary during the first hour after conjugation

[A] RT-PCR analysis of conjugates (AC/AE) treated with nodal inhibitor A-83-01. Early (stage 9-12), later (stage 12-34) and continuous (stage 9-34) treatment after conjugation revealed an absolute requirement for nodal signalling during gastrulation. **[B]** Further refinement of requirement for nodal signalling in conjugates (treatment times shown) revealed nodal is required during the first four hours after conjugation (0-4 h) with an absolute requirement within the first hour (0-1 h). Later treatments have no effect (1-4 h and 2-4 h) compared to controls. **[C]** ImageJ analysis of corresponding PCR.

5.2.2.3 Cardiogenesis requires cell autonomous Activin/Nodal Signalling in the responder

Given the narrow time-frame for requirement of active Nodal signalling these results suggested that embryonic cells need to directly respond to this pathway to adopt cardiac fate. It was unlikely that Nodal was indirectly resulting in cardiac induction by first inducing a secondary factor. However, previous evidence suggested cardiac tissue is induced in VMZ by injection of *Xnr1* and *Cripto*. Mosaic analysis established that the cells expressing *Xnr1* never expressed cardiac markers, and it was concluded that Nodal is inducing cardiac tissue non cell-autonomously (Foley *et al.*, 2007). Furthermore, it was postulated that this inductive event occurs via induction of *Cerberus*, which was also shown to be capable of inducing cardiac markers in VMZ explants, although terminal differentiation markers were never observed (Foley *et al.*, 2007).

Therefore, the cell autonomous requirement for the Nodal pathway in cardiogenesis was examined. This was achieved by mosaic expression of the dominant-negative form of the Activin receptor II (ΔActRIIB; Chang *et al.*, 1997; Hemmati-Brivanlou and Melton, 1992). Activin/Nodal signalling is transduced via binding of ligand to a heterodimeric complex consisting of the activin type II receptor and ALK4/7 (the type I receptor). Like other TGFβs, the type II receptor phosphorylates the type I receptor which then causes phosphorylation of intracellular Smads, in this case Smad2 (Massague, 2008; Shen, 2007). Cripto binding is essential for Nodal signal transduction, achieved by binding of the protein directly to ALK4 via its CFC domain (Yeo and Whitman, 2001). The specificity of TGFβ signalling (i.e. the intracellular Smad proteins activated) is dictated by the Type I receptor, but many different signalling pathways share a common type II receptor. For example, both BMP and Nodal share the ActRIIB receptor and mutually antagonise each other by forming intracellular heterodimers (Reissmann *et al.*, 2001). Composed of only extracellular and transmembrane domains, *ΔActRIIB* has been shown to robustly inhibit Nodal/Activin signalling. *ΔActRIIB* has been shown to prevent mesodermal induction in AC and whole embryos, abolishing expression of early downstream targets (Hemmati-Brivanlou and Melton, 1992; Schulte-Merker *et al.*, 1994; Takahashi *et al.*, 2000). As expected, *ΔActRIIB* has also been shown to inhibit BMP signalling (Hemmati-Brivanlou and Thomsen, 1995), due to it being a common type II receptor. However, since BMP signalling is not required for cardiogenesis in AC/AE (see section 5.4) this reagent was used as cell-autonomous inhibitor of Nodal signalling.

As a result, AC overexpressing *ΔActRIIB* injected in one blastomere of a two-cell embryo were conjugated to AE and analysed by WMISH for cardiac marker expression. Constructs were also co-injected with biotinylated-dextran. This resulted in AC in which the injected responding cells were inhibited from responding to nodal signalling. To determine cell-autonomy, injected blastomeres were developed using FastRed. In conjugates in which one half of the AC were expressing *ΔActRIIB*, cardiac tissue was only ever induced in FastRed negative cells i.e. only in cells with intact activin/Nodal in contrast to controls (figure 5.7c and b respectively). Therefore, Activin/Nodal signalling was required cell-autonomously to induce cardiac tissue. Furthermore, this was confirmed using a constitutively active form of the Activin/Nodal receptor ALK4 (caALK4; Jones *et al.*, 1996). Featuring a single amino acid substitution in the activation domain, *caALK4* results in constitutive ligand-independent activation of activin/nodal signalling. *caALK4* has been shown to be a potent dorsal mesoderm inducer in AC in a dose-dependent manner similar to Activin, confirming it as an Activin receptor. Overexpression of *caALK4* in AC was found to induce cardiac tissue, revealing Nodal signalling is sufficient for cell-autonomous induction (figure 5.7d).

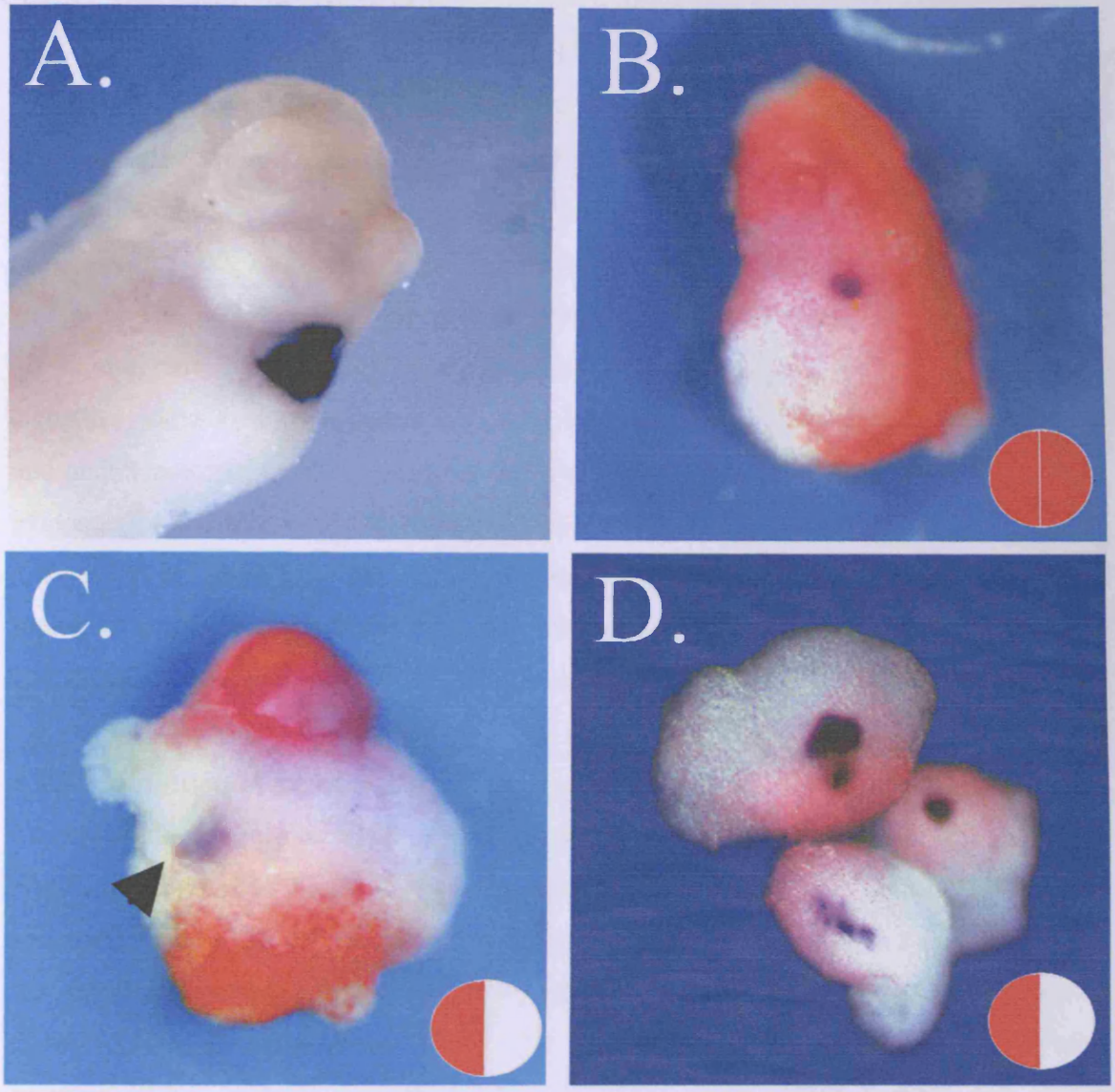


Figure 5.7 – Cell autonomous requirement for nodal signalling in cardiac specification

Nodal/activin pathway is required cell-autonomously for cardiac induction, as revealed by mosaic analysis. **[A]** Stage 34 whole embryo stained for **CTnI**. **[B]** In control AC/AE, AC were injected uniformly with **lineage tracer**, revealed by FastRed staining, showing a single focus of **CTnI** overlapping injected cells (83%, n=12). **[C]** AC/AE in which the nodal and BMP inhibitor, ΔActRIIB, was mosaically expressed in AC (lineage tracer positive) never express CTnI (100% of CTnI+ conjugates, n=11). However, neighbouring cells in the same conjugate with intact nodal signalling (lineage tracer negative) show cardiac marker expression ([black arrowhead]; 46% CTnI+ conjugates, n-=11). **[D]** Similarly, in AC injected with the constitutively active Alk4 (caAlk4) receptor induce cardiac expression cell-autonomously (100% CTnI+ AC, n=12)

5.2.3 The BMP pathway is not required for cardiac specification in AC/AE

BMP proteins have been implicated in multiple aspects of cardiac development in a variety of organisms (Schlange *et al.*, 2000; Schultheiss and Lassar, 1997). In contrast, directed differentiation of ES cells toward a cardiac lineage could not be achieved by BMP addition (Yuasa *et al.*, 2005). Zebrafish *swirl* (bmp2) mutant embryos have abnormal dorso-ventral patterning and lack cardiac progenitors marked by Nkx2.5 expression (Reiter *et al.*, 2001). As in the case of FGF signalling, in *Xenopus* embryos evidence suggests that BMP signalling plays a more apparent role later in cardiac development during morphogenesis and maintenance of cardiac precursors (Shi *et al.*, 2000; Walters *et al.*, 2001). Conflicting evidence for the role of BMP signalling in cardiogenesis therefore exists, exacerbated by difficulties in dissecting out roles for this signalling pathway from its involvement throughout development; the direct implications of perturbed BMP signalling at the time of specification were unclear. These issues were addressed in the AC/AE model.

5.2.3.1 Inhibition of BMP has no effect on cardiogenesis

To investigate the role of BMP in cardiac specification, the ability of the AC to respond to BMP signalling was blocked by injecting a variety of different inhibitory constructs; truncated form of BMP4 receptor I (tBr; Graff *et al.*, 1994), membrane-tethered form of the BMP antagonist *Noggin* (*FlogNog;* Lyle Zimmerman, personal communication), *Cerberus* (Bouwmeester *et al.*, 1996). *tBr* is a mutated form of the type I BMP-specific receptor ALK3, lacking its intracellular serine/threonine kinase domain and acts as a dominant negative shown to block BMP signalling. Overexpression of *tBr* on the ventral side of the embryo results in dorsalisation with expression of dorsal markers such as *Gsc* and formation of neural tissue not normally fated to this region of the embryo (Graff *et al.*, 1994). *Noggin* is a potent dorsalising factor secreted by the Organiser (Smith and Harland, 1992), shown to inhibit BMP2 and 4, and BMP7 to a lesser extent. This is believed to be achieved by direct association with the proteins preventing them interacting with their receptors (Zimmerman *et al.*, 1996). *FlogNog* is a membrane-tethered version of this protein, shown to act as a cell autonomous inhibitor of BMP signalling (Lyle Zimmerman, personal communication). Lastly, *Cerberus* is secreted protein expressed in the AE at gastrulation (Bouwmeester *et al.*, 1996), shown to be a multivalent inhibitor of Nodal, Wnt and BMP signalling (Agius *et al.*, 2000; Piccolo *et al.*, 1999).

AE was conjugated to AC expressing inhibitors of BMP signalling, and cultured until stage 34 for analysis of cardiac marker gene expression. Samples were compared to AE conjugated to uninjected AC as a control (figure 5.7). It was found that inhibition of BMP had no effect upon expression of *MHCα* (figure 5.7a). The effectiveness of these reagents was demonstrated by induction of neural tissue in AC (Bouwmeester *et al.*, 1996; Lamb *et al.*, 1993; Sasai *et al.*, 1995). This would therefore suggest that the BMP pathway is not required for cardiogenesis, at least during the early phases leading up to early differentiation.

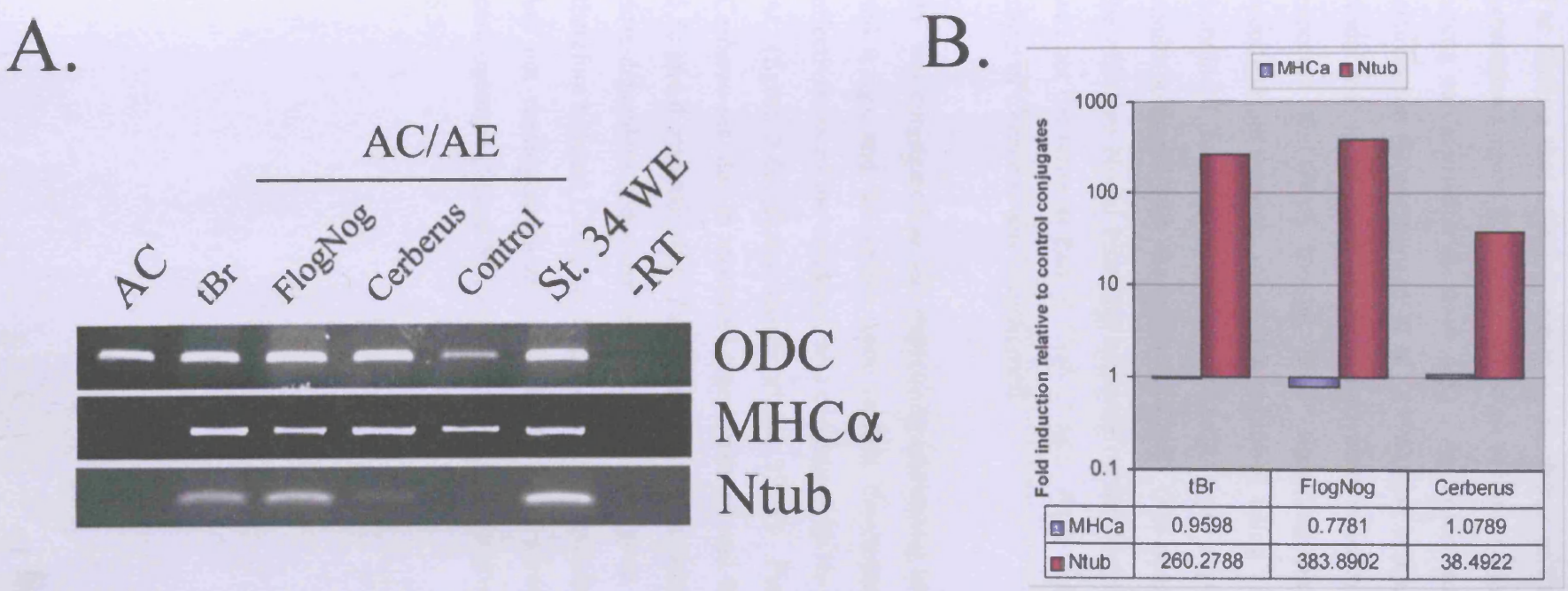


Figure 5.7 – BMP signalling is not required for specification of cardiac precursors in the AC/AE model

[A] Conjugates to anterior endoderm were made with Animal caps (AC) expressing truncated type I BMP receptor (tBR), membrane tethered *Noggin* (FlogNog) or *Cerberus* (1 ng of each mRNA), or uninjected controls. Samples were collected at stage 34 for analysis by RT-PCR, when sibling control embryos (WE) show robust expression of *MHCα* and the neural marker *Ntub*. All inhibitors have no effect on cardiac marker expression. **[B]** Quantification of RT-PCR in [A]. All anti-BMP reagents used were active, as demonstrated by their ability to induce *Ntub*, compared to control samples.

5.2.3.2 Overexpression of Cerberus inhibits cardiogenesis

The finding that overexpression of *Cerberus* in the AC had no effect on cardiac marker expression (figure 5.7) conflicted with evidence showing that inhibition of Nodal signalling blocks cardiogenesis (section 5.3). As described, *Cerberus* is a marker of the AE at gastrulation (Bouwmeester *et al.*, 1996), and is a known multivalent inhibitor of the Wnt, Nodal, and BMP pathways with equivalent efficacy (Piccolo *et al.*, 1999). It was therefore expected that even though BMP signalling has been shown not to effect cardiac specification through inhibition achieved using *tBr* and *FlogNog*, the Nodal inhibitory domain of full-length *Cerberus* would have some effect that would correspond to the results achieved with the truncated *CerS*. One possible explanation for this result was that the effective Nodal blocking capacity of full-length *Cerberus* at the dose injected (1 ng) was not the same as that of *CerS* (1 ng). As a result, a dose-response relationship for the effect of *Cerberus* was investigated.

AE was conjugated to AC expressing increasing concentrations of *Cerberus* mRNA (1, 2, and 4 ng), and the effect upon cardiac development observed (figure 5.8). As before, effectiveness of the construct was confirmed by its ability to induce cement gland tissue in AC (figure 5.8d; Bouwmeester et al., 1996). Furthermore, increasing concentration of *Cerberus* resulted in increased anteriorisation and formation of cement gland tissue (figure 5.8c and d respectively). Interestingly, the increasing dose of the construct also caused a dose-dependent reduction in cardiac marker gene expression (figure 5.8a). This would therefore suggest *Cerberus* displays a dose-dependent action, as it blocked BMP signalling but not cardiogenesis at 1 ng (figure 5.7), while higher concentrations also inhibited cardiogenesis (figure 5.8) to a similar extent observed with the truncated form *CerS* (figure 5.5).

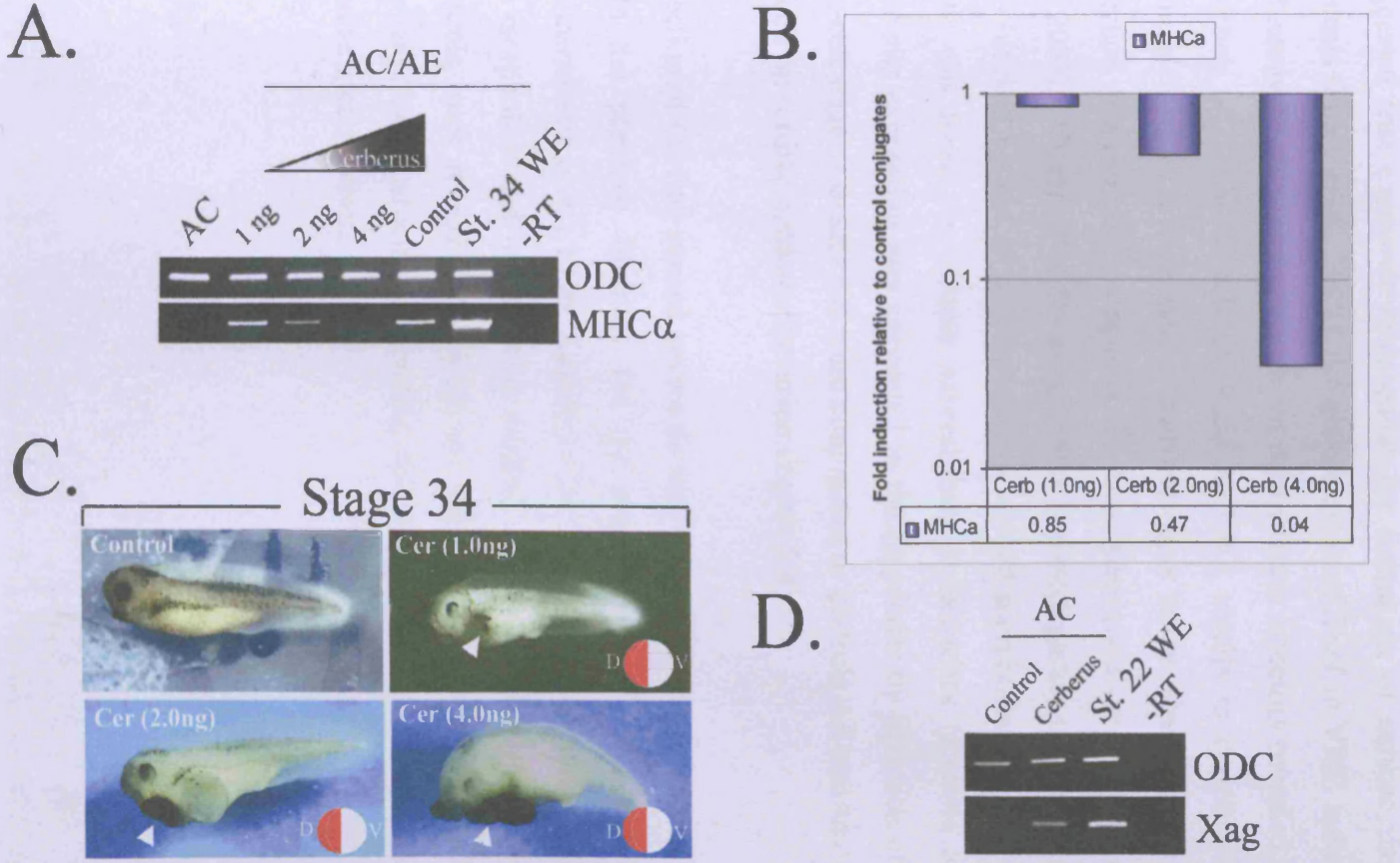


Figure 5.8 – *Cerberus* inhibits cardiogenesis in AC/AE in a dose-dependent manner

Conjugates (AC/AE) were made with animal caps (AC) injected with 1, 2, and 4 ng of *Cerberus* mRNA. Samples were cultured until stage 34 for analysis and compared to uninjected control conjugates. **[A]** RT-PCR analysis for *MHCα* expression shows a dose-dependent inhibition of its expression, with quantification shown in **[B]**. Whole embryo controls for construct activity shows dose-dependent increase in anterior character marked by increased cement gland tissue formation (arrowhead). Stage and location of injection is shown by the schematic in the lower-right corner. **[D]** Molecular confirmation of *Cerberus* induction of cement gland tissue shown by expression of *Xag*.

5.2.3.3 Expression of Cerberus in the responder is not required for cardiac induction

It has been shown that *Cerberus* inhibits cardiogenesis at higher doses, and is likely to be acting via its ability to inhibit the Nodal pathway (figure 5.8). However, it has been previously shown that *Cerberus* is also induced in the responding AC as a result of conjugation (figure 4.12). It would therefore appear contradictory that overexpression of *Cerberus* inhibited cardiac induction. However, the levels of *Cerberus* induced are unlikely to have been sufficient to block Nodal signalling. Furthermore, induction of *Cerberus* is occurring after the requirement for Nodal signalling. Recent evidence has suggested that *Cerberus* is essential for formation of cardiac tissue in *Xenopus* VMZ explants (Foley *et al.*, 2007). Overexpression of *Xnr1* in VMZ induced cardiac tissue non-cell autonomously, and a screen for downstream effectors revealed induction of *Cerberus.* Injection of *Cerberus* mRNA itself in VMZ results in expression of *Nkx2.5,* however terminal cardiac differentiation markers were never observed. It was also shown that injection of a morpholino against *Cerberus* prevented cardiac induction by *Xnr1* (Foley *et al.*, 2007). Therefore to investigate this apparent paradox, *Cerberus* expression in the AC was blocked by use of a previously described morpholino, as that of the aforementioned work was found to contain several base mismatches (Kuroda *et al.*, 2004). Hence, *Cerberus* expression was prevented in the responder by injection of the morpholino at the two-cell stage. *CerMO* AC were conjugated to gastrula AE and analysed at stage 34 for the effects on cardiac marker expression (figure 5.9).

Injection of *CerMO* caused severe defects in anterior development (figure 5.9c), consistent with the previous findings for the requirement of *Cerberus* in head development (Bouwmeester *et al.*, 1996; Kuroda *et al.*, 2004; Schneider and Mercola, 1999). However, the morpholino had no effect on cardiac marker gene expression compared to uninjected controls, even at higher doses (figure 5.9a). This would suggest that *Cerberus* does not mediate Nodal induction of cardiac fate in AC/AE and its expression is not essential for cardiac specification.

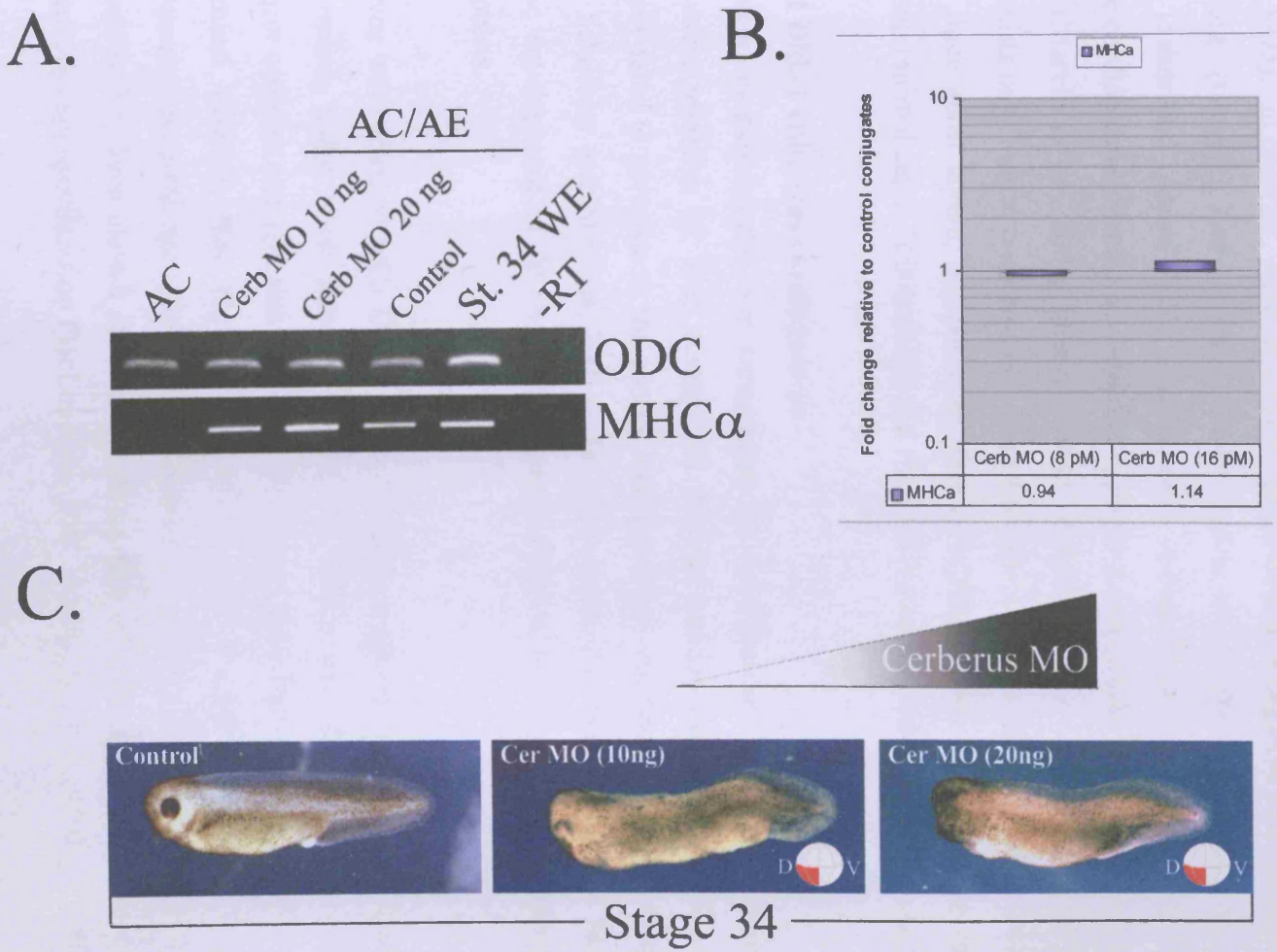


Figure 5.9 – *Cerberus* expression is not required for the specification of cardiac fate

Knockdown of *Cerberus* expression was achieved by injection of the translational blocking morpholino (Kuroda *et al.*, 2004). Conjugates (AC/AE) were made with animal caps (AC) injected with two different doses of MO, and compared to control uninjected AC/AE. **[A]** Analysis of stage 34 AC/AE showed *Cerb* MO has no effect on expression of terminal cardiac differentiation markers (*MHCα*). **[B]** Quantification of PCR in [A] normalised to *ODC*, compared to C AC/AE. **[C]** Phenotypic controls of MO injected embryos, showing dose-dependent anterior defects. Stage and location of injection is indicated in the lower-right corner

5.2.4 Wnt/β-catenin signalling inhibits cardiogenesis but does not block specification

While BMP, FGF and the Nodal/Activin pathways are all implicated in promoting cardiac induction, there is evidence that canonical Wnt signalling blocks and Wnt antagonism stimulates cardiogenesis in chick and *Xenopus* embryos (Marvin *et al.*, 2001; Schneider and Mercola, 2001). Contradictory evidence has suggested an involvement for both Wnt signalling and its inhibition in cardiogenesis (Eisenberg and Eisenberg, 2007). A positive role for the Wnt pathway in cardiac specification has come from work in invertebrates (Wu *et al.*, 1995). In contrast, work in vertebrates has shown a negative role for Wnt/β-catenin signalling (Garriock and Drysdale, 2003; Marvin *et al.*, 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001). Furthermore, it was shown that Wnt antagonism could initiate cardiac development in complex explants not normally fated to give rise to heart tissue (Marvin *et al.*, 2001; Schneider and Mercola, 2001). However, given that Wnt antagonists only induce cardiogenesis from complex explants (VMZs), which contain parts of all three germ layers, but not from simple explants, such as pluripotent prospective ectoderm (animal caps), it is unlikely that they are instructive factors for cardiogenesis.

5.2.4.1 Dkk-1 enhances cardiogenesis

In vertebrates particularly, Wnt antagonism has been deemed important in cardiogenesis with overexpression of Wnt antagonists driving cardiac differentiation in tissue not normally fated to give rise to the cardiac lineage (Foley and Mercola, 2005; Marvin *et al.*, 2001; Schneider and Mercola, 2001). To begin to address the role of Wnt signalling in cardiac development in AC/AE, AE were conjugated to AC cells overexpressing Wnt antagonists.

Embryos were injected with *Dkk-1* and a dominant negative form of the Wnt-dependent transcription factor t-cell enhancer 3 (*TCF3*), which normally forms a transcriptional enhancer complex with β-catenin to drive Wnt target gene expression. Δ*TCF3* contains an N-terminal deletion that prevents interaction with β-catenin, and therefore blocks subsequent transcriptional activation (Molenaar *et al.*, 1996). Overexpression of Δ*TCF3* in the embryo has been shown to block β-catenin induced axis duplication, and also block endogenous axis specification (McLin *et al.*, 2007; Molenaar *et al.*, 1996). Wnt suppressed

AC were conjugated to wildtype AE as described, and cultured until stage 34 for analysis of cardiac marker expression (figure 5.10).

Overexpression of the Wnt antagonist *Dkk-1* resulted in enhancement of cardiac marker expression with a 4-fold increase in levels of *MHCα* (figure 5.10a and b respectively). However, injection of the dominant negative TCF3 construct had no effect on cardiogenesis in AC/AE but the construct was active as it prevented axis specification in WE phenotypic controls (figure 5.10e). *Dkk-1* has been shown to induce ectopic cardiac marker expression in explants of non-cardiogenic mesoderm (Marvin *et al.*, 2001; Schneider and Mercola, 2001), and has similarly been shown to enhance cardiac marker expression in *GATA4* injected AC (Latinkic *et al.*, 2003). Recent evidence has suggested that *Dkk-1* however may harbour a novel cardiac inducing capacity independent of its role in inhibition of Wnt/β-catenin signalling (Foley *et al.*, 2006), localised to its N-terminal domain (Korol *et al.*, 2008). This inducing role for *Dkk-1* was not found to be the case in AC/AE, as co-injection of *CerS* and *Dkk-1* showed no expression of *MHCα* (figure 5.10c). The construct was active as ascertained by its robust induction of the cement gland marker *Xag* in AC, and anteriorisation of the embryo (figure 5.10d and e respectively). Hence, *Dkk-1* cannot induce cardiac tissue in the absence of Nodal signalling and is unlikely to act instructively.

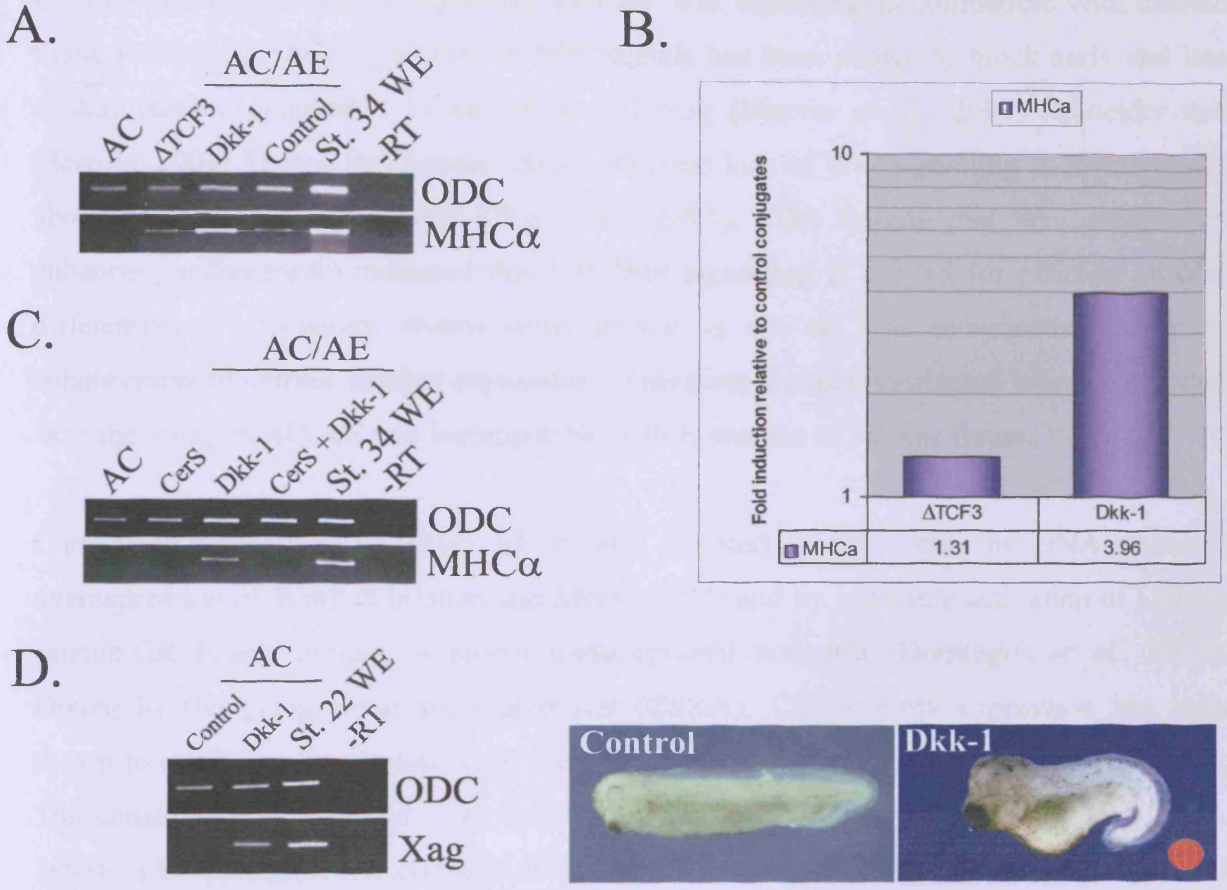


Figure 5.10 – Dkk-1 enhances cardiogenesis but cannot act independently of Nodal signalling
Conjugates (AC/AE) were made to animal caps (AC) injected with the *Wnt* antagonists *dkk-1* and *ΔTCF3* (1 ng of each) and the effects upon cardiogenesis compared to uninjected controls. **[A]** RT-PCR analysis for *MHCα* expression shows an increase in expression upon injection of *Dkk-1*, quantified in **[B]**. **[C]** Coinjection of *Dkk-1* however, cannot rescue the knockdown of cardiac marker expression observed upon nodal inhibition, achieved by uniform injection of *CerS* (1 ng) in the AC. **[D]** Activity of *Dkk-1* constructs was confirmed by its ability to induce cement glands in AC, ascertained by expression of *Xag* absent from control AC. **[E]** Phenotypic confirmation of anteriorisation induced upon uniform injection of *Dkk-1*.

5.2.4.2 Elevated Wnt/β-catenin signalling blocks cardiogenesis

Conflicting evidence exists regarding whether Wnt signalling is compatible with cardiac tissue formation. Overexpression of Wnt ligands has been shown to block early and late cardiac marker expression in the chick and frog (Marvin *et al.*, 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001), whereas loss of Wnt signalling in invertebrates abolishes cardiac differentiation (Wu *et al.*, 1995). The finding that Wnt antagonism enhances cardiogenesis indicated that low Wnt signalling is needed for efficient cardiac differentiation. However, results were unclear as not all Wnt antagonists resulted in enhancement of cardiac marker expression. Therefore it was investigated whether elevated Wnt signalling in AC/AE was incompatible with formation of cardiac tissue.

Conjugates were made in which AC exhibit elevated Wnt/β-catenin by DNA-mediated overexpression of Wnt8 (Christian and Moon, 1993) and by inducible activation of LEF-β-catenin-GR fusion protein, a potent transcriptional activator (Domingos *et al.*, 2001). Driven by the cytoskeletal actin promoter (CSKA), CSKA-Wnt8 expression has been shown to result in posteriorisation of the embryo characteristic of elevated Wnt signalling. The construct is transcribed from MBT into early tailbud stages (Christian and Moon, 1993). LEF-β-catenin-GR consists of the hormone binding domain of the glucocorticoid receptor (GR) fused to the Lef1 DNA-binding domain and C-terminal transactivation domain of β-catenin (Vleminckx *et al.*, 1999). The GR fusion protein is activated by administration of the readily diffusible steroid ligand dexamethasone (dex) at any developmental stage. These fusion proteins are known to be highly specific, stable conditionally active constructs (Mattioni *et al.*, 1994). Furthermore, the β-catenin clone has a truncated version of the transactivation domain, lacking the phosphorylation sites essential for targeted degradation induced by GSK3β. In the presence of dex, this fusion protein constitutively activates the β-catenin target genes *Siamois* and *Xnr3*, and causes axis duplication in the whole embryo independently of endogenous β-catenin (Domingos *et al.*, 2001; Vleminckx *et al.*, 1999). Conjugates were therefore analysed at stage 34 for any apparent effects on cardiogenesis (figure 5.11).

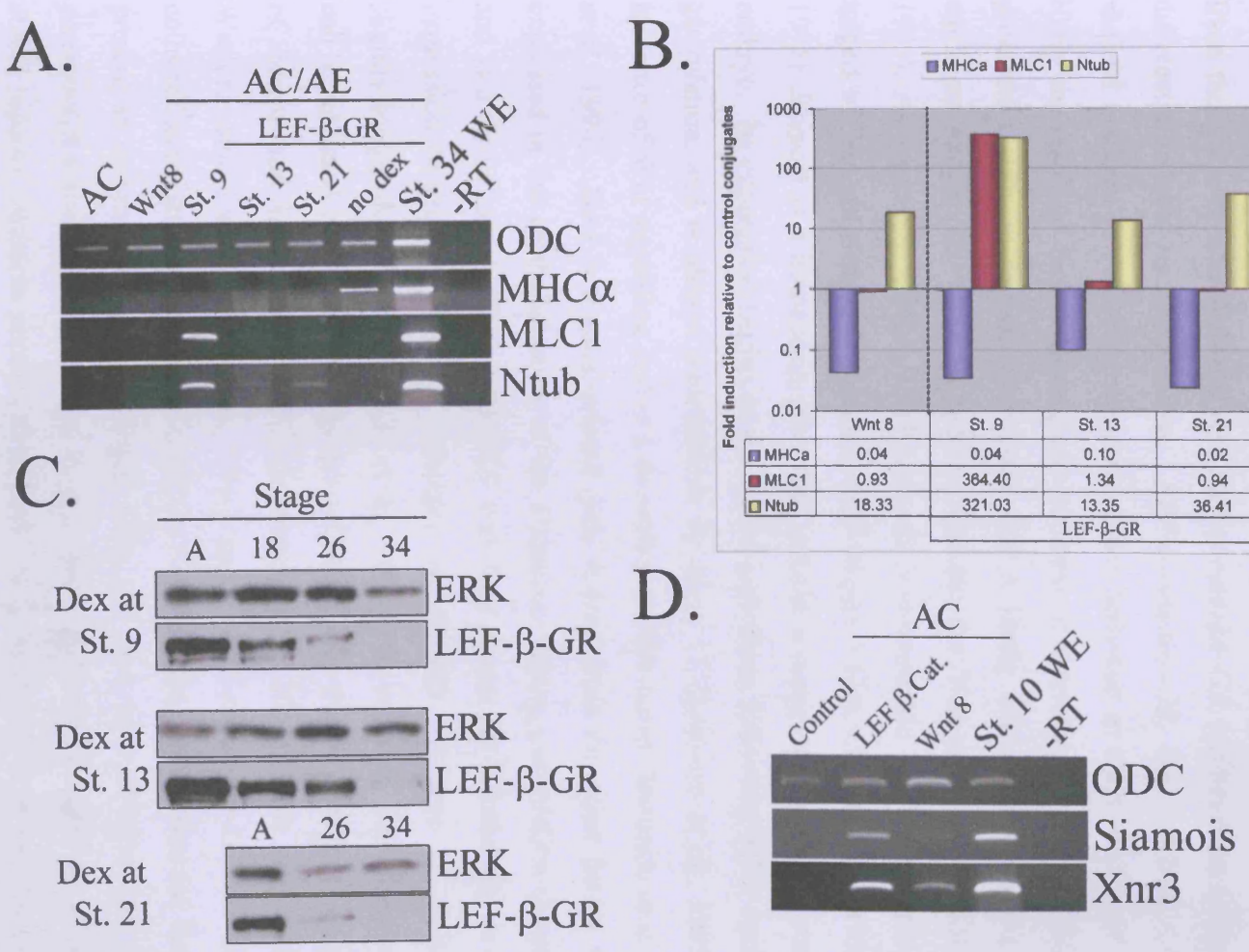


Figure 5.11 – Elevated Wnt/β-catenin signalling opposes cardiogenesis in AC/AE

Anterior endoderm was conjugated to animal caps (AC) injected with either CSKA-Wnt8, or LEF-β-catenin-GR activated with dexamethasone at indicated stages. **[A]** RT-PCR analysis of stage 34 conjugates shows all forms of *Wnt* activation abolishes cardiogenesis, as indicated by expression of *MHCα*. Control conjugates were injected with LEF-β-catenin-GR that was never activated. Samples were also analysed for the muscle and neural markers, *MLC1* and *Ntub* respectively. **[B]** ImageJ quantification of PCR in [A]. **[C]** The expression of the LEF-β-catenin-GR fusion protein was analyzed by Western blotting for HA tag. The protein was expressed efficiently at all stages at which dexamethasone was added and was found to persist for many hours. Protein levels were normalised for endogenous ERK. **[D]** Activity of Wnt constructs was confirmed by injection into AC, collected at stage 10 for analysis. Both constructs show induction of the Wnt downstream targets, *Xnr3* and *Siamois*, with LEF-β-catenin-GR a more potent activator.

Overexpression of CSKA-Wnt8 and *Lef-β-catenin-GR* mRNA (activated at stage 9 at the time of conjugation) efficiently blocked cardiogenesis with a severe reduction in *MHCα* expression (figure 5.11a). This suggested Wnt/β-catenin signalling is incompatible with cardiogenesis. Utilising the conditionally inducible nature of *LEF-β-catenin–GR*, it was possible to activate Wnt signalling at progressively later stages through cardiac development and observe the effect on cardiac marker expression. Interestingly, activation of Wnt signalling shortly after gastrulation (stage 13) or during early tailbud stages (stage 21) similarly effected cardiac marker expression. In contrast, in the absence of dex AC/AE showed robust expression of *MHCα* providing a control for the inducibility of the construct.

Even though both CSKA-Wnt8 and *LEF-β-catenin–GR* inhibited cardiogenesis, they had differential effects on myogenesis. *LEF-β-catenin–GR,* but not CSKA-Wnt8, induced skeletal markers in AC/AE conjugates when activated at stage 9 (figure 5.11a); This is likely the result of their differing effectiveness at activating Wnt signalling. The CSKA promoter results in mosaic expression and it likely takes several hours to accumulate sufficient exogenous Wnt8 protein to activate the Wnt pathway (Christian and Moon, 1993). As expected, CSKA-Wnt8 induced low-moderate levels of expression of direct targets of Wnt pathway, *Siamois* and *Xnr3* (figure 5.11d; Carnac et al., 1996; Smith et al., 1995). *Siamois* is a homeobox gene that induces a complete secondary axis in the whole embryo. Its expression begins in the dorsal endoderm following MBT and peaks prior to gastrulation, and is almost undetectable by stage 11 (Lemaire *et al.*, 1995). It is a key mediator of Wnt signalling and is a downstream Wnt target (Brannon *et al.*, 1997; Carnac *et al.*, 1996). *Xnr3* is a nodal-related gene distinct from the other family members. It is expressed in the epithelial layer of the Organiser during gastrulation (Smith *et al.*, 1995) and is a direct target of the LEF/TCF and Wnt signalling (McKendry *et al.*, 1997). Its expression begins at MBT and although undetectable by stage 12, it is expressed for slightly longer that *Siamois* (Smith *et al.*, 1995). In contrast, *LEF-β-catenin–GR* protein can be activated very efficiently by dex addition when desired, leading to strong expression of *Siamois* and *Xnr3* (figure 5.11d; Domingos et al., 2001). This was further shown by a Western blot for Haemaglutinin (HA)-tagged *LEF-β-catenin–GR* in explant samples collected at the time of dex addition (figure 5.11c). This showed that the fusion protein was present at approximately equal levels from the time of activation and that the *LEF-β-catenin–GR* protein is very stable, as previously indicated (Domingos *et al.*, 2001). This stable inducer therefore persists to activate its target genes for many hours (figure 5.11d).

This finding therefore suggests that myogenesis requires relatively high levels of Wnt/β-catenin signalling in the AC/AE model. In addition, previous findings suggest overexpression of Wnt in AC is insufficient for skeletal muscle and neural marker expression (Christian and Moon, 1993; Domingos *et al.*, 2001), and the competence is lost after stage 9. The results obtained here are consistent with these findings.

5.2.4.3 Wnt/β-catenin does not block specification of cardiac precursors

It has been shown that overexpression of Wnt in the AC blocked expression of terminal cardiac differentiation. However as previously stated, this form of analysis is retrospective and there is significant time-delay between the time of cardiac specification and expression of contractile proteins (Mohun and Sparrow, 1997). It was therefore difficult to determine at which point Wnt signalling was incompatible with cardiogenesis. To gain further insight, conjugates overexpressing *LEF-β-catenin-GR* were analysed at stage 18 for expression of the early cardiac markers *Nkx2.5* and *Tbx5* (figure 5.12). Despite completely abolishing expression of terminal cardiac differentiation markers, elevated Wnt/β-catenin signalling had no effect on expression of early markers. This suggests Wnt signalling does not block specification of cardiac precursors, but in contrast is incompatible with cardiogenesis following its induction.

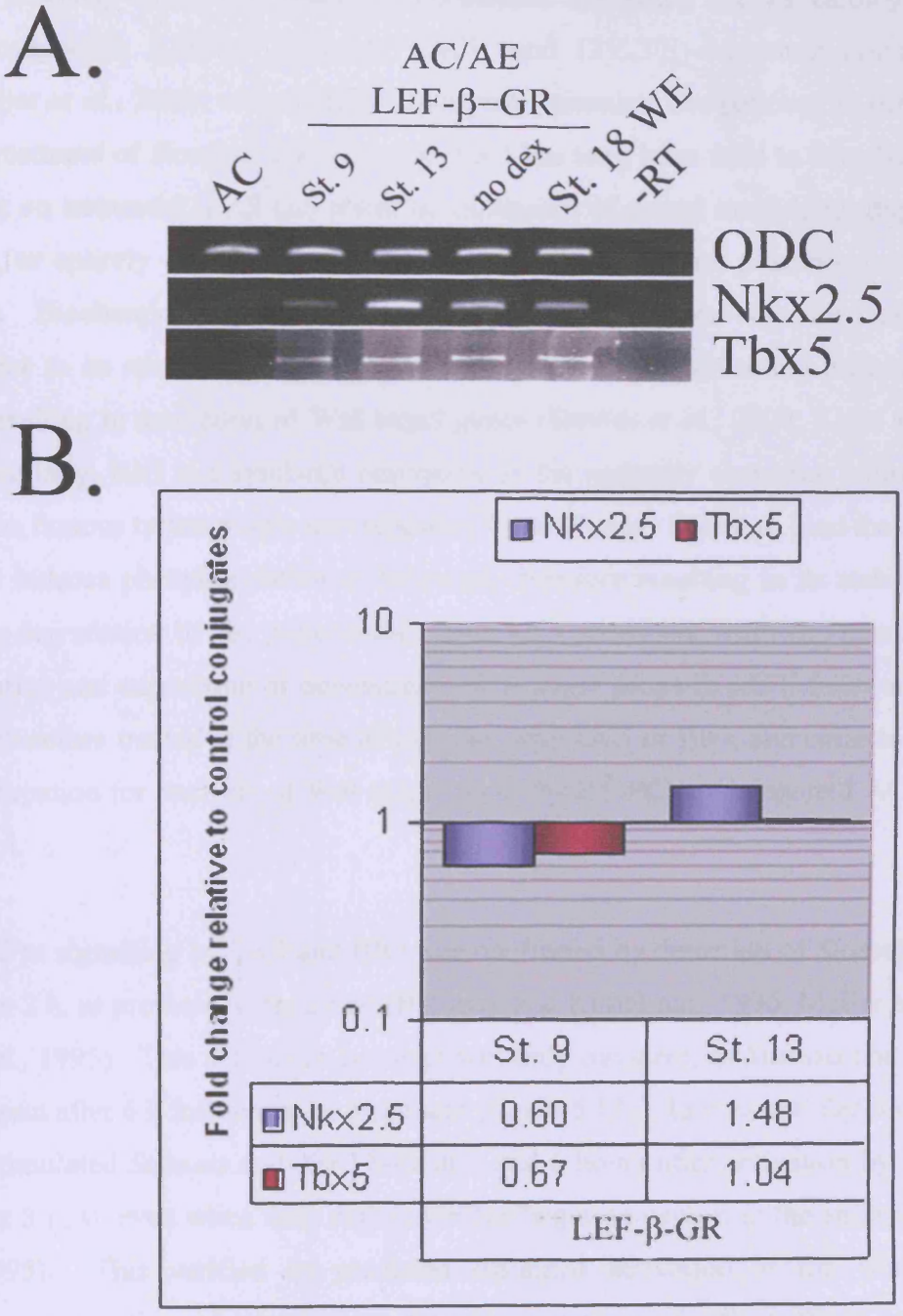


Figure 5.12 – Increased Wnt signalling does not block cardiac specification

Conjugates (AC/AE) of anterior endoderm were made to animal caps overexpressing LEF-β-catenin-GR, activated by addition of dex at the time of conjugation or slightly later. **[A]** RT-PCR analysis of stage 18 AC/AE shows overexpression of the Wnt construct has no effect on the expression of the early cardiac markers *Nkx2.5* and *Tbx5*, compared to control conjugates where the construct was never activated. **[B]** Gel densitometry readings of *ODC* normalised gene expression compared to control conjugates

5.2.4.4 Wnt/β-catenin signalling inhibits cardiogenesis after specification

To more precisely determine when Wnt/β-catenin signalling blocks cardiogenesis, the soluble compounds Lithium Chloride (LiCl) and (2'Z,3'E)-6-bromoindirubin-3'-oxime (BIO; Meijer *et al.*, 2003) were used to transiently stimulate the pathway at different time points. Treatment of *Xenopus* embryos with LiCl has long been used to dorsalise embryos. Depending on treatment, LiCl can result in expansion of dorsal mesoderm, duplication of body axis, or entirely dorsalised embryos lacking all ventral and posterior tissue (Sive *et al.*, 2000). Biochemical studies revealed the phenotypes observed upon treatment with LiCl are due to its specific direct inhibition of GSK3β, thus preventing degradation of β-catenin resulting in activation of Wnt target genes (Davies *et al.*, 2000; Klein and Melton, 1996). Similarly, BIO is a synthetic derivative of the naturally occurring indirubin that is found in the famous tyrian purple dye produced by molluscs. Found to bind the ATP site of GSK3β, it reduces phosphorylation of β-catenin therefore resulting in its stabilisation and preventing degradation by the proteasome. Like LiCl, treatment with BIO results in dorso-anteriorisation and expression of downstream Wnt target genes in AC (Meijer *et al.*, 2003). AC were therefore treated at the time of excision with LiCl or BIO, and collected 2 h and 6 h after extirpation for analysis of Wnt target genes by RT-PCR. Uninjected AC were used as a control.

Elevated Wnt signalling by LiCl and BIO was confirmed by detection of *Siamois* and *Xnr3* in AC after 2 h, as previously described (Brannon and Kimelman, 1996; Meijer *et al.*, 2003; Smith *et al.*, 1995). This activation however was only transient, as *Siamois* and *Xnr3* were largely absent after 6 h following the treatment (figure 5.13a). In contrast, *Lef-β-catenin-GR* strongly stimulated *Siamois* and *Xnr3* both at 2 and 6 hours after activation by addition of dex (figure 5.13a), even when their expression has begun to decline in the embryo (Lemaire *et al.*, 1995). This verified its predicted sustained activation of the Wnt pathway. Furthermore, activation of target genes by overexpression of CSKA-Wnt8 led to lower levels of expression that were however maintained for a longer period of development.

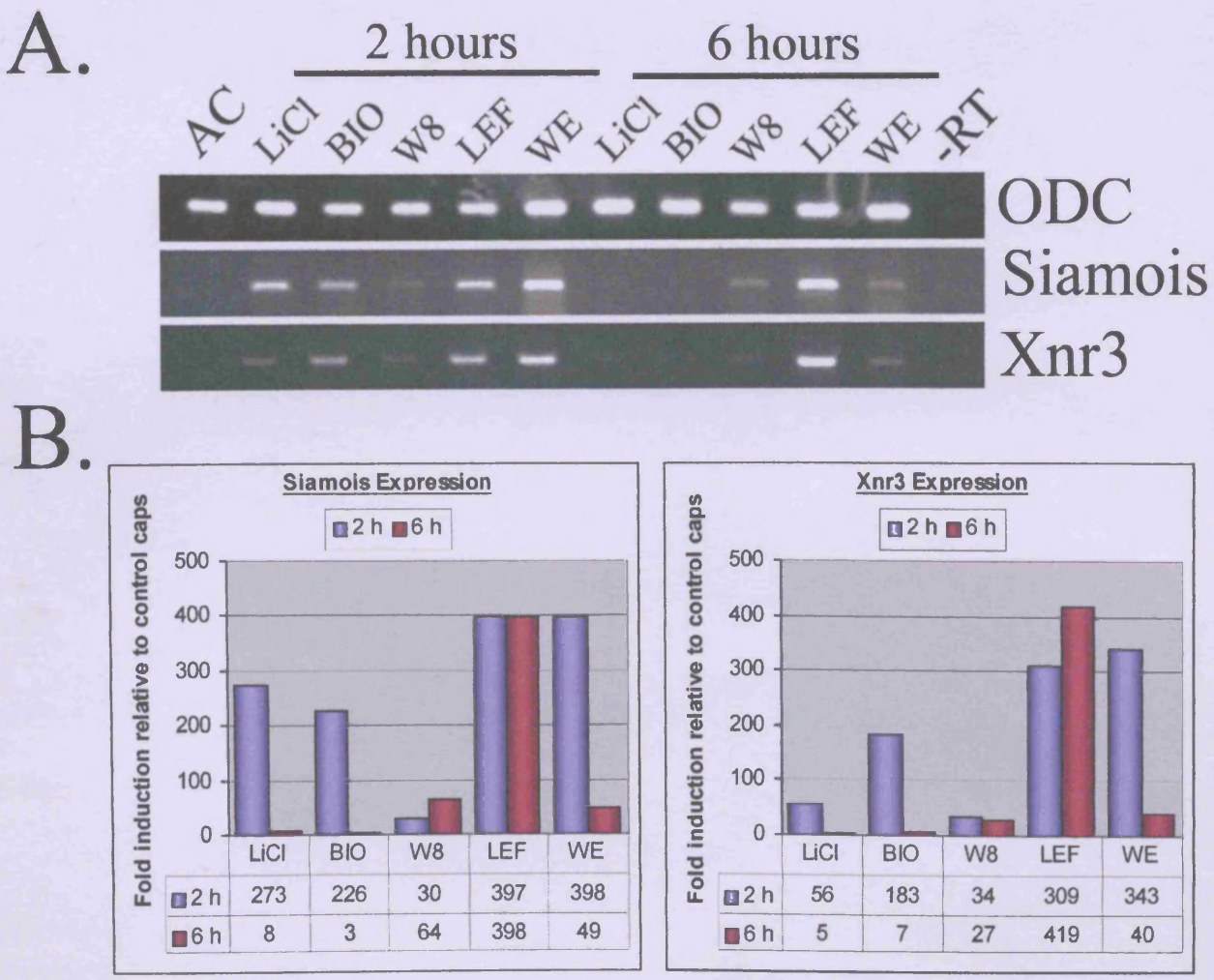


Figure 5.13 – Analysis of the effects of various agonists on the Wnt/β-catenin pathway

Elevated Wnt signalling in animal caps (AC) was achieved by treatment of uninjected AC with LiCl or BIO, or upon addition of dexamethasone to LEF-β-catenin-GR injected AC. Samples were then analysed for expression of the Wnt target genes *Siamois* and *Xnr3*. **[A]** **RT**-PCR analysis of treated AC collected 2 h and 6 h after Wnt activation. LiCl and BIO cause strong activation of target genes at 2 h, but only transiently as their expression is not detectable after 6 h. Overexpression of Wnt8 causes a low-level sustained activation of target genes. Conversely, LEF-β-catenin-GR causes strong, prolonged activation of the Wnt pathway, even as levels in the whole embryo begin to decrease. **[B]** ImageJ analysis of *Siamois* and *Xnr3* expression observed in [A], normalised to ODC

The findings that these GSK3β inhibitors provide transient activation of Wnt signalling provided the opportunity to determine the exact time-point during cardiogenesis when the Wnt pathway is incompatible with cardiac tissue formation. Early LiCl treatment at the time of conjugation had no effect on cardiogenesis while it stimulated myogenesis (figure 5.14a and c). In contrast, treatments at stage 21 and 24 blocked cardiac differentiation. Once cardiac differentiation commences at stage 27 however, LiCl had no effect on *MHCα* expression (figure 5.14a and c). The effects of LiCl were mirrored by BIO, confirming their specificity (figure 5.14b and d). It can therefore be concluded that Wnt/β-catenin signalling has no effect on cardiac specification, but rather it antagonises cardiogenesis just prior to the onset of terminal differentiation.

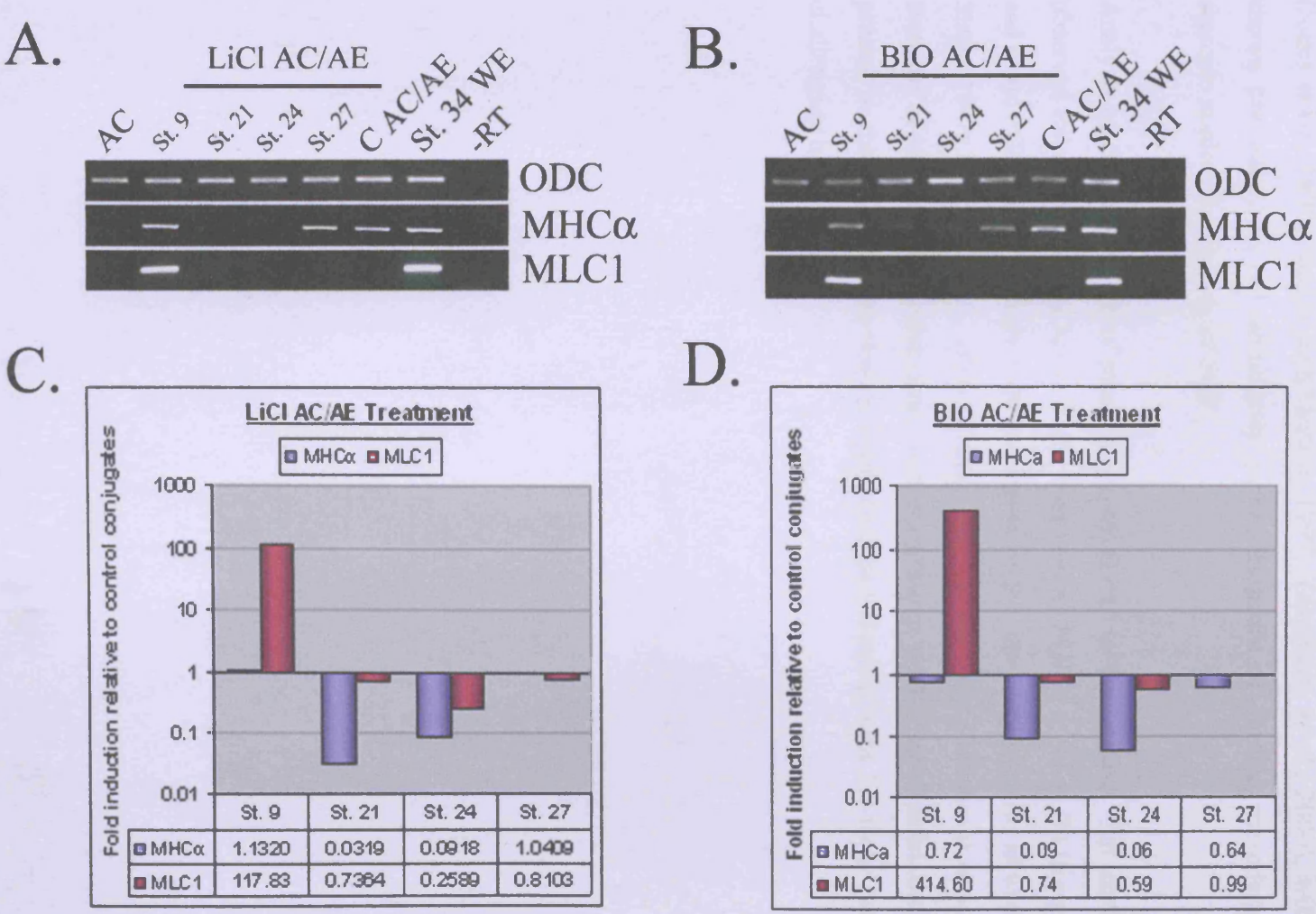


Figure 5.14 – Wnt signalling is incompatible with cardiogenesis after specification prior to the onset of terminal differentiation

Examination into the sensitivity window of AC/AE to elevated Wnt signalling was achieved using LiCl and BIO. **[A]** Uninjected conjugates were treated with LiCl (0.3 M) for 10 min at indicated stages. RT-PCR analysis reveals treatment at stage 9 does not block *MHCα* expression but does induce skeletal muscle (*MLC1*). Later treatment at stage 21/24 however, blocks cardiac marker expression but has no effect at stage 27. **[B]** Similarly, treatment with BIO (8 μM) for 20 min showed an effect on cardiogenesis at stages 21/24, but not at stage 9 or 27. **[C, D]** ImageJ quantification of PCRs shown in [A, B] respectively.

5.2.5 Identification of ligands responsible for induction

The finding that both FGF and Nodal appear to be inducing cardiac tissue led to investigation into which FGF/Nodal ligands may be good candidates for specification. Multiple members of the FGF and Nodal families are known to be expressed in early gastrula anterior endoderm. All Xnr ligands except Xnr3 show appropriate expression (Jones *et al.*, 1995; Joseph and Melton, 1997; Takahashi *et al.*, 2000), with Xnr-1,-2,-5, shown previously to be particularly potent inducers of cardiac mesoderm. A similar scenario applies for ligands of FGF.

Analysis of AE at the time of excision revealed this to be the case. An incomplete survey observed the expression of Xnr1, 2 and 5 as well as FGF3, 4, 9 and 20 (figure 5.15). Xnr4 and 6 and FGF8 are also likely to be expressed by the anterior endoderm as well (Christen and Slack, 1997; Fletcher et al., 2006; Joseph and Melton, 1997; Takahashi et al., 2000). The scenario of considerable redundancy and complexity within both Nodal and FGF families present in the AE indicates that defining cardiac-inducing roles for these factors will be a challenging task.

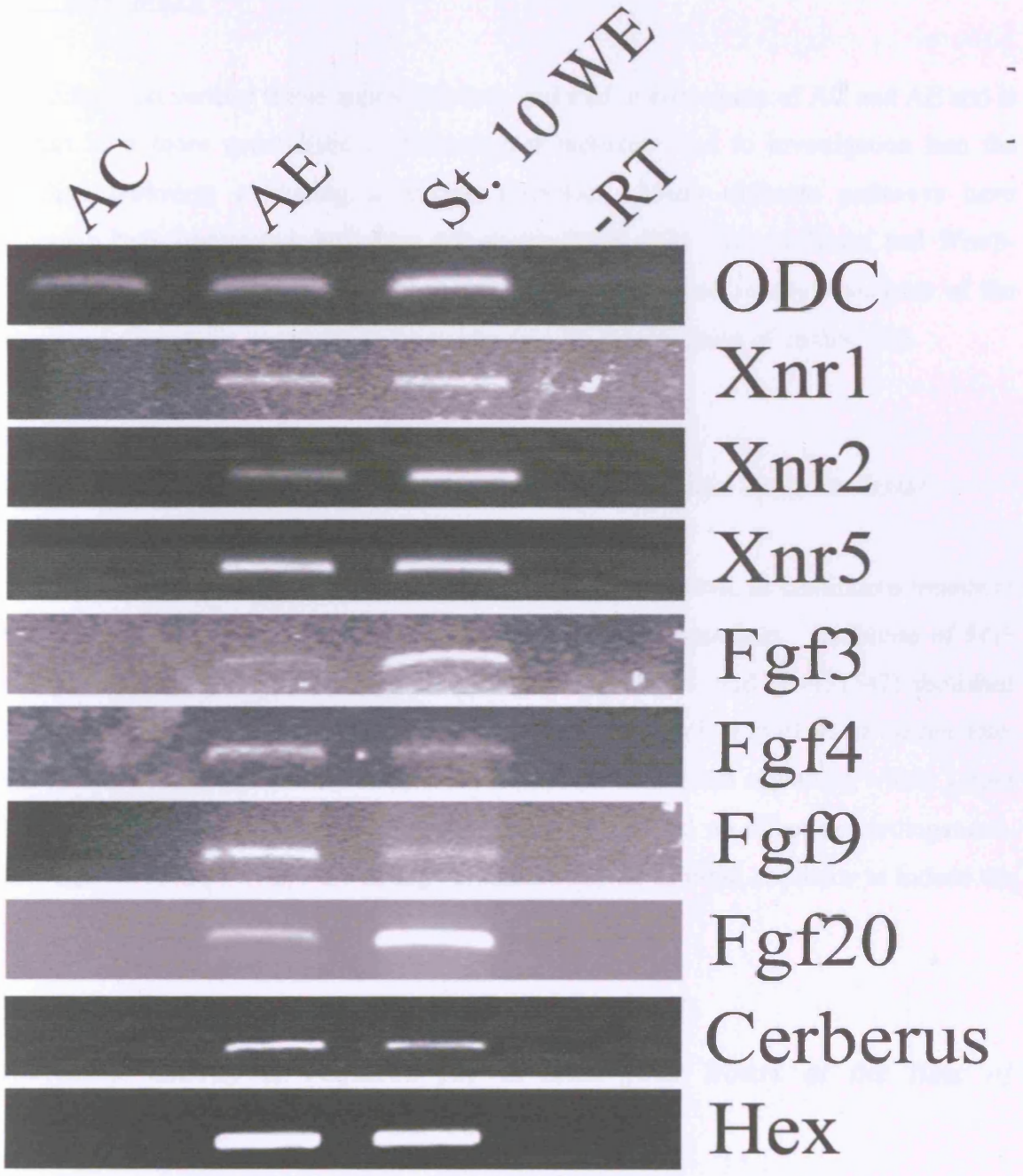


Figure 5.15 – Anterior endoderm explants express several Nodal and FGF genes

Anterior endoderm explants were analyzed for expression of indicated genes immediately after excision. All genes tested are expressed in stage 10 anterior endoderm. Anterior character of endoderm explants was confirmed by expression of Cerberus and Hex.

5.3 Summary

The finding that cardiac tissue appears directly induced in conjugates of AC and AE and is not part of a more generalised mesendodermal induction, led to investigation into the signalling pathways mediating a cardiac response. Many different pathways have previously been implicated, including that of the BMP, FGF, Activin/Nodal and Wnt/β-catenin pathways. Although not exhaustive, analysis provided in depth analysis of the signalling factors most suggested to be responsible for specification of cardiac fate.

5.3.1 FGF and Nodal signalling are required to induce cardiac tissue

A positive requirement for Nodal and FGF signalling was shown, as continuous treatment with inhibitors blocked expression of early and late cardiac markers. Inhibition of FGF (using SU5402 and ΔFGFR1) and Nodal (using CerS, A-83-01, and SB-431542) abolished expression of *Nkx2.5, Tbx5, MHCα*, and *MLC2,* early and terminal markers of cardiac fate. Timed inhibition revealed a requirement for active FGF and Nodal signalling within 1 hour after conjugation, and inhibiting these pathways after this had no effect on cardiogenesis. This suggests both pathways are acting simultaneously or in rapid sequence to induce the cardiac program.

5.3.2 ERK activity is required for at least four hours at the time of specification

By using the chemical inhibitor U0126, it was shown that FGF signalling in cardiac induction is likely to be mediated by the Ras/MAPK transduction pathway. Treatment with this inhibitor from the time of conjugation abolished cardiac marker expression. However, timed inhibition of ERK activation in conjugates revealed a different requirement to that of FGF signalling at the level of the receptor. Whereas blocking FGFR1 was required within the first hour, it was shown that activated ERK was required for the first 4 hours for maximal levels of cardiac marker expression. Treatment of conjugates with U0126 at 0-2 h or 2-4 h after conjugation only permitted induction of cardiac fate to a level that was

approximately half as efficient as control conjugates. One implication of this result is that ERK activity is required during the entire 4 hour period after conjugation for maximal cardiogenesis (relative to control AC/AE explants), in contrast to an absolute requirement of FGF ligand-receptor interaction in the first hour of induction.

5.3.3 BMP is dispensable for cardiac induction

Inhibition of the BMP pathway using a truncated version of the BMP receptor and a modified version of the antagonist Noggin had no effect on terminal cardiac differentiation. *Cerberus,* a multivalent inhibitor of BMP, Wnt and Nodal signalling displayed dose-dependent action. At 1 ng it was unable to block cardiac differentiation but sufficient to inhibit BMP as ascertained by its ability to induce neural tissue in AC. At higher doses however, it blocked cardiac induction through action of its Nodal inhibitory domain. These results however demonstrate that BMP signalling is not required for cardiogenesis in the AC/AE model system, at least during the early phases of differentiation.

5.3.4 Wnt/β-catenin blocks cardiogenesis downstream of specification

In addition to an inductive role for both Nodal and FGF in cardiac induction, it was shown that Wnt/β-catenin signaling does not block specification of precursors as previously described. Firstly, it was shown that overexpression of *dkk-1* enhanced cardiac marker expression. Furthermore, overexpression of Wnt8 DNA or LEF-β-catenin-GR activated at various time points blocked terminal cardiac differentiation. However, further analysis revealed that expression of the early cardiac markers *Nkx2.5* and *Tbx5* were not affected, suggesting the incompatibility of Wnt signaling was not at the time of specification. This led to further investigation into relative effectiveness of different activators of Wnt signaling used in embryology. It was shown that both CSKA Wnt8 and LEF-β-catenin-GR induce downstream Wnt targets for a long sustained period, with LEF-β-catenin-GR being a very potent activator. However, both GSK3β inhibitors (LiCl and BIO) only transiently activate Wnt signaling, with expression of downstream targets almost undetectable six hours after treatment. Therefore transient activation of Wnt signaling in conjugates was

used to determine the exact time point when cardiogenesis was blocked by the pathway. Contrary to previous findings, activation of Wnt at the time of specification did not block cardiogenesis and induction of skeletal tissue was also apparent. However, it was found that activation of Wnt during tailbud stages blocked terminal cardiac differentiation markers and only became compatible just prior to onset of their expression at stage 27. A requirement was therefore shown for suppression, or low activity, of Wnt/β-catenin pathway from late neurula or early tailbud stages until the onset of cardiomyocyte differentiation at late tailbud stages.

CHAPTER 6 - DISCUSSION

6.0 DISCUSSION

Heart development is a much studied area, but the early signalling events resulting in recruitment to the cardiac lineage and the signalling source responsible were poorly defined. Much evidence has suggested a key involvement of the AE in cardiac induction in vertebrates (Nascone and Mercola, 1995; Schultheiss *et al.*, 1995; Sugi and Lough, 1994). However, the details of the role of the AE in inducing cardiac fate are largely unknown. Although deemed necessary for cardiogenesis, it had not been clearly shown whether AE was sufficient. Previously, the myocardial inducing capacity of the endoderm was assayed with tissues normally fated to give rise to heart, therefore not revealing whether signals were instructive and therefore influencing lineage decisions, or permissive and merely promoting already specified tissue. Through modification of a simple induction assay (Nieuwkoop, 1969) the precise role of the AE in the early *Xenopus* embryo and its involvement in cardiogenesis was investigated.

Utilising the model of AE conjugated to pluripotent AC has permitted the investigation into the mechanisms of cardiac development. Unlike other models however, it was possible to directly examine the immediate events of cardiac specification. In contrast to other experimental explants, such as VMZ in *Xenopus* embryos or the lateral and posterior mesoderm explants of the chick, AC/AE does not require modification of pre-existing mesoderm; cardiac mesoderm is induced *de novo* in pluripotent responding embryonic ectoderm from signals emitted from the relevant source (gastrula AE). By directing blastula AC cells toward cardiac induction, a fate that is not normally exhibited by this tissue, the AC/AE conjugation system is a true model of tissue induction and specification. The assay employs conjugation of heterochronic endodermal and ectodermal explants, which makes a direct study of induction feasible even though this interaction does not normally occur. Nonetheless, given that AC/AE conjugates faithfully reproduced virtually every aspect of early cardiogenesis, they are a tractable model for detailed analysis of cardiac induction.

6.1 AC/AE as a Model for Cardiogenesis

6.1.1 Pure AE explants were excised that were stable for long-term culture

In this study, it has been shown that it is possible to isolate the AE at a much earlier time-point than previously described, at a time when cardiogenesis is predicted to occur (Sater and Jacobson, 1990b). This permitted investigation into its role in cardiac fate. Previous results have shown that at stage 10 the endoderm underlies the mesoderm and the cells are in close contact. Indeed previously it was found that AE extirpation was commonly contaminated with mesoderm, making their use much more limited (Horb and Slack, 2001; Sater and Jacobson, 1989). Through analysis of early markers of mesodermal and Organiser fates, namely *Xbra* and *Gsc*, it was shown that using the described method of dissection the AE was isolated with no expression of such genes (figure 3.5). Thus it is indeed possible to isolate the AE, and it was also shown that this tissue was stable enough to be cultured until a late stage in development (stage 34). Furthermore, the signalling capacity of the excised endoderm was confirmed by its ability to induce anterior character, both in complex explants and naïve AC tissue (figure 3.6 and 3.7 respectively), as previously described (Jones *et al.*, 1995).

6.1.2 The AE is both necessary and sufficient for cardiogenesis

The potential of the AE to initiate cardiogenesis was tested by its ability upon conjugation to re-specify pluripotent AC. It was shown that the AE induced cardiac fate in the cells of the responding AC, with expression of early (*Nkx2.5*) and of terminal cardiac differentiation markers (*MLC2, CTnI, MHCα*; figure 3.8). In addition, samples showed expression of late regional specific cardiac markers which are associated with mature heart formation (*Irx4, MLC1v, Tbx18*; figure 3.9). There is also evidence to suggest that the cardiac tissue induced exhibited anterior-posterior patterning, with expression of markers restricted to ventricle and atrial tissue of the myocardium, and also tissue of the proepicardium. This would imply that cardiac tissue has been induced and to some degree patterned, with evidence of some simple rudimentary organisation and A-P diversification.

In some cases, formation of spontaneously beating tissue resulted, confirming specification and maintenance of terminally differentiated cardiomyocytes. In previous models of heart development in *Xenopus,* either utilising the AC model or via complex MZ explants, formation of beating tissue was also noted (Latinkic *et al.*, 2003; Logan and Mohun, 1993; Pandur *et al.*, 2002; Schneider and Mercola, 2001). However in all previous studies, the incidence of beating tissue was low and the heart tissue formed, although well-differentiated, showed poor structural organisation. In contrast, it would appear that in the model of the AC/AE there is induction of rhythmical contracting tissue with evidence of further cellular diversification and organisation comparable to the mature heart. This provides overwhelming evidence that the AE is essential for specification of the cardiac mesoderm, with resultant formation of fairly complex heart tissue requiring a further involvement of the AE (Muslin and Williams, 1991; Tonegawa *et al.*, 1996). This could be addressed by examining regional specific marker expression in AC peeled from the AE (discussed in 6.2.1).

6.1.3 Anterior endoderm induces cardiac tissue in absence of the Organiser

Previous evidence has suggested that the AE is required to induce cardiogenesis in conjunction with a dorsalising influence believed to be provided by the Organiser (Nascone and Mercola, 1995; Sater and Jacobson, 1990b). In the AC/AE model however, early explants of AE showed no expression of *Gsc*, a known marker of the Organiser at the time of extirpation. Therefore, the resultant induction of cardiac tissue upon conjugation to the AC was occurring in the absence of defined Organiser tissue, consistent with findings in the chick (Schultheiss *et al.*, 1995). Taken together, these results would strongly suggest that the AE is solely responsible for the inductive events leading to the induction of cardiac fate. The Organiser however, is still likely to be required in the whole embryo for cardiac specification to occur. The AC responder is a naïve tissue that is capable of being driven toward multiple fates. This is very different to the mesoderm in *vivo* adjacent to the endoderm, whose competence to adopt a cardiac fate is very different. There is strong evidence to suggest the Organiser is required to dorsalise the mesoderm, for example through secretion of the BMP antagonist *Noggin* to permit the mesoderm to acquire competence to respond to the inductive events of the AE (Smith *et al.*, 1993). This may

explain the findings of Nascone and Mercola (1995), who showed that the AE alone could not induce ventral mesoderm to adopt cardiac fate. However, the failure of ventral mesoderm to form cardiac tissue can only partly be explained by the lack of involvement of the Organiser in this region of the embryo. Furthermore, it has been shown that regions of posterior endoderm are incapable of inducing cardiac tissue. This is consistent with previous mesoderm induction experiments that showed that dorsal mesoderm (although cardiac tissue was not investigated) can only be induced by more anterior regions of the vegetal pole (Dale and Slack, 1987). This would suggest that the failure of ventral mesoderm to adopt cardiac fate in the embryo is due to both lack of competence of the responding tissue, and lack of cardiac inducing potential of posterior endoderm.

6.1.4 The AE inducing centre acts specifically and directly to induce cardiogenesis

In addition to cardiac tissue AE induced markers of other cell types involved in cardiovascular development, namely endothelium, macrophages, smooth muscle, and blood. Importantly, AC/AE explants were free from skeletal muscle (and neural tissue) demonstrating that AE was not inducing cardiac tissue as a part of general mesoderm induction. Further evidence for this came from the peeled AC assay, in which AC peeled after 2 h of exposure expressed a similar profile of cell fates as observed in whole AC/AE. At early stages, analysis of a range of mesendodermal genes showed expression of markers of mesoderm and endoderm (figure 4.12). This raises two potential areas of debate. Firstly, it was shown that markers of the Organiser (*Gsc* and *Chordin*) were induced in the AC raising the possibility that this was indirectly contributing to cardiac induction. Although it cannot be conclusively said not to be the case without further analysis, this is unlikely given the short time frame in which the specification events are occurring. Furthermore, expression of a few markers related to the Organiser does not imply it is induced, as this process itself is much more complex (section 1.1) and importantly requires Wnt signalling unlike AE-mediated cardiogenesis (discussed below). Secondly, induction of endodermal tissue in the AC raised the possibility that the endoderm may have contributed toward cardiogenesis. Conjugates in which animal cap explants were expressing dominant-negative Sox17β protein induced higher level of cardiac markers

(figure 4.13), suggesting that in AC/AE explants Sox17-dependent endoderm opposes cardiogenesis. A similar result was previously described in animal caps in which mesoderm and endoderm were induced by *GATA4* (Latinkic *et al.*, 2003). Likewise, blocking *Hex*-dependent endoderm using morpholino oligonucleotides and Hex VP16 had no effect on cardiac marker expression. This confirmed cardiac tissue was likely induced directly independent of induced endoderm.

Further evidence that induction of cardiac tissue is not a result of general mesoderm induction was obtained by the finding that markers of posterior endoderm (*Xpo* and *Vent2;* figure 4.12b) were not induced, and the system is therefore selective for anterior fates. Specification upon conjugation to AE appears to be much more selective for cardiovascular cell fates, in a similar manner to the *GATA4* cardiac model (Latinkic *et al.*, 2003). Injection of *GATA4* however, is still capable of inducing cardiac tissue as late as neurula stages, a time by which cardiac specification in *vivo* has already occurred (Sater and Jacobson, 1989). Although obviously important in establishing the cardiac lineage, it is therefore very likely *GATA4* is acting downstream of the actual specification events that occur in *vivo.* In contrast, the AC/AE model utilises an inducing tissue from the embryo at the time when cardiac specification is thought to occur, and its inductive capacity is therefore a true reflection of the specification events of the embryo.

The lack of investigation into cardiac fate in previous conjugation models (Dale and Slack, 1987; Nieuwkoop, 1969), led to the investigation of whether standard Nieuwkoop sandwiches could induce cardiac tissue (figure 4.4). It was found that this was not the case but in contrast, there was strong expression of muscle and neural markers as previously described (Dale and Slack, 1987; Nieuwkoop, 1969). The rationale was that this difference in cardiac inducing capacity may have arisen from the difference in nature due to age of the inducing tissues (stage 8.5 vegetal pole versus stage 10.25 AE). However, when vegetal poles were explanted and aged to the same developmental stage as the AE, there was still no expression of cardiac markers. This comparison demonstrates that the two inducing tissues produce distinct signals (and a possible explanation is discussed in section 6.2.2), and that the blastula vegetal pole requires further interaction with the embryo to become competent to form cardiac tissue. Formally, it is possible that in *vivo* the posterior endoderm inhibits (or restricts) the anterior vegetal pole in cardiogenesis which is normally

restricted by the action of the Organiser. However, excising this tissue at blastula stages therefore prevents this restriction and so the anterior region is inhibited.

6.1.5 The inductive response to the AE is localised and non-uniform

Formation of small foci of cardiac tissue in the AC in close cellular proximity to the cells of the AE, and the failure of the PE to induce cardiogenesis led to the hypothesis that the cardiac inducing nature of the AE was not uniform. Dissection of AE explants into more anterior and posterior portions prior to conjugation resulted in enrichment of the expression of cardiac markers in more anterior explants (figure 4.6). This was in addition to a substantial decrease in cardiac induction on posterior AE explants. The apparent increase in expression in anterior explants was associated with more than one focus of cardiac marker expression, which upon analysis correlated with an increased anterior character of inducer as determined by expression of the homeobox transcription factor *Hex* (figure 4.7). It has previously been documented that *Hex* expression confers anterior identity. Interestingly, at gastrula stages *Hex* is expressed in a wedge-like domain extending from the dorsal lip to the floor of the blastocoel (Jones *et al.*, 1999). This encompasses an area reminiscent of the 30-45° of DMZ to the left and right of the dorsal midline, known to be the heart forming region of the embryo (Sater and Jacobson, 1990b). This apparent localisation of the inducer was reiterated by formation of several smaller foci upon dissociation of the AE prior to conjugation. The localisation of cardiac muscle formation appears similar to that previously reported in the induction of muscle (Gurdon, 1989). In conjugates of vegetal and animal pole it was documented that muscle cells were always formed in one or two groups along the interface between the two tissues. Furthermore, the muscle cells were always near the inducer, requiring close proximity but not cell-cell contact. Cardiac induction therefore appears to follow a similar closely regulated process, ensuring localised induction of this particular cell fate. Such a response is thought to arise from the fact that only a limited number of cells are competent to respond to a certain signal. These cells must then be within close enough proximity to the inducer to receive sufficient inductive signal(s), above a certain threshold to direct a response during the competent phase (Gurdon, 1987). In the context of the AC/AE model this is closely reflected by the results regarding inducing capacity and competence discussed below.

6.2 Competence of Cardiogenesis and Generation of the Cardiogenic Signal

6.2.1 Competence and inducing capacity for cardiac fate is restricted to early gastrulation

Previous evidence has indicated that cardiac specification occurs during gastrulation (Nascone and Mercola, 1995; Sater and Jacobson, 1989). Further to this, it was shown that cardiac specification occurred during early gastrulation (figure 4.8). Aging of AE explants prior to conjugation showed that peak expression of terminal cardiac markers resulted upon conjugation of early gastrula AE, with a sharp decline in inducing capacity of the AE by stage 11. It is therefore apparent that the cardiac inducing capacity of the AE is very short-lived, and supports previous findings suggesting that vegetal regions can only induce mesoderm up until stage 11 at the latest, with peak induction prior to this (Gurdon *et al.*, 1985; Jones and Woodland, 1987). Furthermore in the peeled AC assay, it was found contact with the AE was only necessary for up to 2 h to induce levels of cardiac marker expression comparable to whole AC/AE (figure 4.10). In contrast to cardiomyocytes, the induction of other cell fates in peeled AC was achieved after 1 hour of exposure, suggesting that different mechanisms operate to induce those fates. Cardiac induction was therefore occurring soon after contact with the AE and continued involvement of the AE was not required beyond specification. It is still possible however, that formation of the more complex structures observed in control conjugates (i.e. formation of contractile tissue and expression of chamber specific markers) does require involvement of AE (Muslin and Williams, 1991; Tonegawa *et al.*, 1996). Further analysis of pAC for more advanced cardiac markers such as *MLC1v* or *Irx4*, and morphology is required to determine the roles of the AE beyond specification. This analysis was outside the scope of this study.

Responding tissues themselves are also restricted to adopt particular fates, and the AC is known to have defined competence to certain inductive events. For example, competence of AC to respond to FGF signalling is lost by stage 10, whereas responsiveness to activin treatment is possible up until stage 11 (Green *et al.*, 1990). Furthermore, conjugation experiments revealed that AC can only respond to mesodermal signals from vegetal regions up until stage 10.5 (Gurdon *et al.*, 1985; Jones and Woodland, 1987). In support of this, the

AC were found to only be competent to respond to cardiac inducing signals of the AE up until stage 10 with a marked decrease in cardiac marker expression even by this stage. In addition, the AC were shown to already express markers of their default epidermal cell fate by the end of gastrulation. This also suggests that the AE is not capable of re-directing this already specified tissue, in contrast to *GATA4* which could weakly specify cardiac tissue in AC as late as stage 18 (Latinkic *et al.*, 2003).

6.2.2 Generation of AE as the inducing tissue of cardiac fate

The main focus of this thesis was detailed characterisation of the events from the initial specification of cardiac precursors. However brief insight into how this cardiogenic signal is generated was revealed by several experiments. It has already been concluded that stage 9 vegetal pole cannot induce cardiac tissue (figure 4.4). It would therefore appear that the vegetal region acquires cardiac inducing capacity at some time between stage 9 and the onset of gastrulation. One possible explanation could be the correlation between inducing capacity and *Hex* expression (figure 4.7), known to be localised in the AE at gastrulation (Newman *et al.*, 1997). Already shown to confer anterior signalling properties (Brickman *et al.*, 2000; Jones *et al.*, 1999), it also appears that the heart inducing region of the early embryo is closely associated with the *Hex* expressing domain at gastrulation (Jones *et al.*, 1999; Sater and Jacobson, 1990b). This may suggest that the *Hex* expressing nature of the AE is essential to confer its ability to induce cardiac tissue, further supporting the suggested role of *Hex* in cardiogenesis downstream of *Wnt* antagonism (Foley and Mercola, 2005). However, work by Zorn *et al.* (1999) revealed that the anterior endomesoderm is regionally specified by blastula stages. Analysis of dorsal endoderm at stage 10 excised at stage 8 revealed expression of markers of patterning, including *Hex* which are not expressed at the time of excision. However, *Hex* expression in isochronic and heterochronic vegetal conjugates (figure 4.4b) was not observed, in contrast to that of AC/AE. This was also the case for the anterior endodermal marker *Cerberus*, which has also been implicated in cardiac specification (Foley *et al.*, 2007) and acts downstream of *Hex* (Zorn *et al.*, 1999). Overexpression of the dominant negative *Hex* construct and *Hex* MO in the responder did not have any effect on cardiogenesis (figure 4.13a), which rules out a direct involvement of *Hex* in mediating cardiogenic signal in the AC of AC/AE conjugates. However, blocking

Hex (or *Cerberus*) expression in the AE prevented expression of terminal cardiac differentiation markers (figure 4.13c). This is likely to be due to indirect effects upon patterning, altering the inducing properties of the AE. It has been shown that *Hex* is required during blastula stages to amplify Wnt signalling by repressing the Wnt antagonist Tle4, necessary for induction of the Nieuwkoop centre. Hex therefore normally, via its role in induction of the Nieuwkoop centre, contributes to anterior identity by promoting expression of *Xnrs,* and subsequently *Cerberus* (Zamparini *et al.*, 2006). This would therefore suggest that the anterior character of the endoderm as a result of expression of these markers (Brickman *et al.*, 2000; Jones *et al.*, 1999; Smithers and Jones, 2002) is essential to confer cardiac inducing capacity in the AE. The lack of anterior character in stage 8 blastula vegetal pole (as demonstrated by lack of *Hex* expression; figure 4.4b) accounts for its failure to induce cardiac tissue. It has been acknowledged that the previous findings stating Wnt antagonism induces cardiac fate (at least directly) were inaccurate (reviewed by Eisenberg and Eisenberg, 2007; Foley and Mercola, 2005). It was concluded that evidence actually suggests Wnt antagonism acts at the level of patterning the endoderm to result in secretion of a cardiac inducing factor. This is consistent with the results presented here. The direct requirement for *Hex* in inducing this factor however is unresolved. This can also be similarly said of *Cerberus,* which was suggested to act downstream of Xnr signalling to induce cardiac tissue in a distinct pathway from that of the Hex-Wnt antagonism pathway (Foley *et al.*, 2006; Foley *et al.*, 2007). This requirement in AC/AE however has been shown not to occur, as overexpression of Cerberus MO in the responder had no effect, and therefore Cerberus is not required downstream of Nodal for cardiogenesis (figure 5.9). It has actually been shown however that *Hex* and *Cerberus* may act in the same pathway, with both induced by Xnrs and evidence also shows that *Cerberus* is indeed induced by *Hex* itself (Brickman *et al.*, 2000; Jones *et al.*, 1999; Zorn *et al.*, 1999). Furthermore, *Hex* has actually been shown to be required for *Cerberus* expression (Zamparini *et al.*, 2006). It is possible that *Cerberus* is mediating the action of *Hex* in generating cardiac inducing activity, and it would be interesting to determine if this is actually the case. This could be addressed by analysis the expression of *Hex* in *Cerberus* MO overexpressing AE and whether *Hex* overexpression could rescue the block in cardiogenesis. Furthermore, this could be extended to determine if the reciprocal is also apparent, and whether *Cerberus* overexpression could rescue the *Hex* knockdown.

6.2.3 Wnt signalling regulates myogenic Vs cardiogenic activity of the AE

AE induces cardiac tissue but not skeletal muscle, whereas blastula stage vegetal pole have been shown to have opposing inducing activity (figure 4.4). The failure of AE to induce skeletal muscle is likely due to the absence of Wnt signalling. It is known that Wnt/β-catenin alone cannot induce skeletal muscle tissue in AC (Christian and Moon, 1993) but can modify induction of mesoderm by other signalling pathways (Christian *et al.*, 1992; Sokol and Melton, 1992). This was shown in AC/AE by overexpressing Wnt using LEF-β-catenin-GR (activated at stage 9), LiCl or BIO, resulting in induction of skeletal muscle (figure 5.11, 5.14). This therefore shows that induction of heart and skeletal muscle is not mutually exclusive, and is likely to be regulated by finely tuned Wnt signalling in the embryo by the Organiser, as it is required for skeletal muscle formation but not cardiac tissue.

6.3 FGF and Nodal mediate Cardiac Specification independently of Wnt/β-catenin Signalling

6.3.1 FGF and Nodal signalling as inducers of cardiac fate

Investigation into the early signalling of cardiac specification in AC/AE showed an absolute requirement for both FGF and Nodal signalling in the first hour of conjugation. Activin/Nodal signalling has been previously implicated in cardiac development either indirectly in zebrafish and mouse (Gritsman *et al.*, 1999; Reiter *et al.*, 2001; Zhou *et al.*, 1993) or by its ability to induce cardiac tissue *in vitro* in *Xenopus* (Ariizumi *et al.*, 2003; Foley *et al.*, 2007; Logan and Mohun, 1993; Takahashi *et al.*, 2000). Results presented here confirm these findings, and actually show that Nodal is acting from the AE to induce cardiac tissue. Our results and previous findings however differ at the level of specificity; previous models in which Nodal/Activin signals directed cardiac differentiation appeared to be the result of a more general mesoderm induction (Ariizumi *et al.*, 2003; Logan and Mohun, 1993)). A possible explanation for this may lie with the level and duration of signal received. It has been shown that Nodal specification of mesodermal derivatives occurs sequentially from late blastula stages (with particular reference to induction of

somites, notochord, blood, and then heart). It was suggested that this change in cell fate is dictated by the level of Nodal signalling received, which ultimately depended upon the length of exposure to Nodal signalling with more dorsal derivatives requiring a longer duration of signalling (Hagos and Dougan, 2007). It is known that Nodal competence is lost by stage 11, due to prevention of nuclear localisation of its intracellular mediator Smad2 by phosphorylation (Grimm and Gurdon, 2002). Due to this loss in competence of Nodal signalling, it is therefore possible that the gastrula AE exposes the AC to a short duration of Nodal signalling (and therefore level), which is sufficient to generate heart tissue but not more dorsal derivatives, such as muscle.

However, this investigation has showed for the first time that FGF is essential for cardiac induction in *Xenopus.* Previous evidence for the role of FGF in cardiogenesis has come from work in the chick and zebrafish developmental systems (Alsan and Schultheiss, 2002; Marques *et al.*, 2008; Reifers *et al.*, 2000). Hitherto, its role was thought to be much more permissive acting in synergy with BMP (Barron *et al.*, 2000; Lough *et al.*, 1996). Using a variety of carefully controlled inhibitory compounds (and constructs) of FGF signalling, it can be concluded that FGF is required immediately or in very rapid sequence with that of Nodal to induce cardiac mesoderm. This is in contrast to the previously proposed relationship between these factors in general mesoderm induction in which mesodermal precursors were thought to be merely maintained by FGF signaling in a positive feedback mechanism (Cornell *et al.*, 1995; LaBonne and Whitman, 1994; Schulte-Merker and Smith, 1995). A direct role for FGF is supported by recent evidence showing that FGF signalling is not only required for maintenance of mesodermal precursors but is also required for their induction (Fletcher and Harland, 2008).

Whereas FGF receptor signaling was shown to be important during the first hour after conjugation, sustained ERK activation was required for the first four hours for maximal cardiac output. This supports previous evidence showing the requirement for MAPK signaling in mesoderm induction (LaBonne *et al.*, 1995). The exact mechanism by which the duration of activated ERK dictates the level of cardiac response requires much further study. It has been shown that in cell culture models that different levels of ERK activation can regulate a fundamental difference in cell fate decision, such as cell differentiation or proliferation. The mechanisms are however complex, thought to involve many receptor interactions, scaffold proteins, and interplay between multiple intracellular kinases and

phosphatases (reviewed by Murphy and Blenis, 2006). The exact mechanism by which embryonic cells interpret sustained ERK activity in the context of the cardiac specification program requires much further study.

Exactly how both these inducing pathways act to drive cardiac differentiation is intriguing. Both pathways have been shown to act quickly, and it is likely that synergism between them is required. It is likely however, that a cooperative effect contributes to the specificity of this cardiac model as it is known that FGF can modify the mesoderm response of TGFβ signaling (Cordenonsi *et al.*, 2007; Cornell *et al.*, 1995). Once such mechanisms are understood, it will permit reconstitution of the AE derived signal verifying sufficiency of these signals to drive cardiac specification. A recent potential mechanism was suggested to be mediated by tumour suppressor protein 53 (p53) phosphorylation (Cordenonsi *et al.*, 2007). It was shown that FGF enhanced Activin mesoderm induction, but this could not occur in p53 depleted AC. Furthermore, it was shown that this action may be mediated by interaction with Smad proteins, which upon treatment with FGF inhibitors was prevented (Cordenonsi *et al.*, 2007). How such interactions affect cardiac fate are unknown, and elucidating such mechanisms will be further complicated by the evidence showing several FGF and Nodal ligands may be mediating AE-derived cardiac induction (figure 5.15).

6.3.2 BMP signalling is dispensable for cardiac specification in AC/AE explants

Blocking BMP signaling had no effects upon cardiac specification consistent with previous findings in *Xenopus*, in which a requirement only at later stages of development was shown (Breckenridge *et al.*, 2001; Shi *et al.*, 2000; Walters *et al.*, 2001). It is possible that blocking BMP may have affected expression of more complex cardiac markers (such as *MLC1v* and *Irx4*) by perturbing morphogenesis. A later involvement is likely, and further investigation into this is required as it was not addressed in this study. This role for BMP however, is in contrast to evidence proposed in the chick and fish (Alsan and Schultheiss, 2002; Ladd *et al.*, 1998; Lough *et al.*, 1996; Reiter *et al.*, 2001). This potentially could be attributed to species-specific difference between the different vertebrates. For example, in chick and fish BMP expression is known to co-localise with cardiac precursors at

specification (Reiter *et al.*, 2001; Schultheiss *et al.*, 1997), which may account for their synergy with FGF (Barron *et al.*, 2000; Lough *et al.*, 1996). Such expression is not apparent in *Xenopus*.

6.3.3 Wnt signalling blocks cardiogenesis but not at specification

Sustained activation of the Wnt/β-catenin signalling was found to block cardiogenesis, but it was found that transient activation of the pathway using LiCl or BIO did not block specification. A Wnt-sensitive window was however revealed from stage 21 until stage 27, during which time activation of Wnt signalling blocked cardiogenesis. This therefore shows a requirement for suppression or low activity of Wnt signalling for a period of several hours prior to the onset of differentiation. This is in contrast to previous findings showing that Wnt signalling blocked cardiac specification (Marvin *et al.*, 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001). Previous work using chick and *Xenopus* embryos and explants has shown that overexpression of Wnt3 or Wnt8 inhibits cardiogenesis (Marvin *et al.*, 2001; Schneider and Mercola, 2001), and this result is comparable to our finding with Wnt8 DNA injection. In previous work, the time window of sensitivity of cardiogenesis to the Wnt/β-catenin pathway could not be determined because of sustained activation of the pathway. Furthermore, our results are consistent with a biphasic role for Wnt signalling in ES cells (Naito *et al.*, 2006; Ueno *et al.*, 2007). Utilising inducible promoters to activate or repress Wnts at different time points, they found Wnt was required before gastrulation and not afterward. It was concluded that Wnt signalling is required to specify the precardiac mesoderm activating a feedback loop resulting in subsequent inhibition of Wnt promoting cardiac differentiation (Ueno *et al.*, 2007). One difference between this model and that of our own is that we show no evidence for stimulation of cardiac development by Wnt/β-catenin signalling. This is likely because our inducer (AE) has been generated during D-V axis specification by Wnt signalling (Zorn *et al.*, 1999) prior to the time we excise it; in ES cells cardiac inducing signals have to be generated from the beginning to mimic germ layer specification.

In agreement with previous work, Dkk-1 enhancement of cardiogenesis was observed (Latinkic *et al.*, 2003; Marvin *et al.*, 2001; Schneider and Mercola, 2001). However, this

was not consistent for all Wnt antagonists as the intracellular Wnt inhibitor, ΔTCF3, had no effect (figure 5.10). It is possible that this novel enhancement by Dkk-1 is due to its suggested role to activate non-canonical signalling by via JNK (Caneparo *et al.*, 2007; Korol *et al.*, 2008), which has previously been implicated in cardiac development (Pandur *et al.*, 2002). Furthermore, this apparent enhancement by Dkk-1 does not completely exclude the early involvement of Wnt on cardiac specification. Without analysis into temporal requirements for Wnt antagonism on cardiac readout in similar manner to that performed for Wnt activation, firm conclusions regarding this cannot be made. However, it is clear that previous suggested roles for Wnt antagonism as a direct inducer of cardiac specification are misleading, which is further supported failure of Dkk-1 to rescue the block in cardiac expression due to truncated Cerberus expression in conjugates (figure 5.10). It is more accurate to describe their involvement as indirect with their role more likely placed before specification of precursors (Manisastry *et al.*, 2006). The contribution of Wnt antagonism to the specificity of cardiac induction (in this model) is however apparent, as the apparent lack of Wnt in AC/AE accounts for the failure of control conjugates to express skeletal tissue markers. This was further exemplified by overexpression of Xnr5 in AC which induced both skeletal and cardiac tissue, of which the expression of muscle markers prevented by co-injection with *Dkk-1* (preliminary data).

6.4 Induction of Liver and Pancreas in Conjugates

In addition to inducing cardiovascular fates in the AC by the AE, conjugates also showed expression of foregut fates (figure 4.3). The AE when cultured in isolation only expressed general endodermal markers, such as *Hex, Sox17,* and *Edd.* However, analysis of conjugates revealed expression of regional specific markers of liver (*For1*, LFABP) and pancreas (Pdx1, XPDIp). This suggested that only upon conjugation can these markers be expressed, confirming previous finding that state a requirement for mesoderm for regional specification (Horb and Slack, 2001). The failure of peeled AC to show such markers suggests it is the AE that is specified and not induction of these markers in the AC, although cell-autonomy is required. This however offers a novel assay for the characterisation of the mechanisms involved in liver and pancreas specification.

6.5 Future Perspectives

The AC/AE conjugation system has therefore permitted dissection of the specification events of cardiogenesis. To gain further insight into the early signalling events of cardiac specification, we began by investigating some of the key pathways implicated in various different models. The list however is not exhaustive as several other pathways and molecules have suggested roles in cardiogenesis. For example, it has been suggested that both the notch/delta and hedgehog pathways may have roles in early cardiac development (Miazga and McLaughlin, 2009; Thomas *et al.*, 2008 respectively). In addition, exactly if and how the GATA transcriptional regulator family are interlinked within this early signalling network would be very interesting, as they known to have important roles in cardiac development (Peterkin *et al.*, 2005). Furthermore, it will be of interest to determine the mechanisms by which FGF and Nodal interact to drive cardiac development in a restricted manner. How exactly the level of ERK activation is interpreted, and the mechanisms by which it elicits alternative cellular fates is intriguing. Only with such understanding of the more complex mechanisms involved in specification is it possible to accurately reconstitute the cardiogenic program in absence of the inducing tissue. This will then permit the genomic response of cardiac specification to be elucidated, potentially identifying key cardiac regulatory determinants.

CHAPTER 7 - BIBLIOGRAPHY

7.0 BIBLIOGRAPHY

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APPENDIX

APPENDIX I

Accepted Correspondence - Development

In their recent publication, Afouda et al have reported expression of a cardiomyocyte-specific marker MHCα in GATA4-GR injected animal caps at stages 11 and 20 (Fig. 3 in (Afouda et al., 2008)). This unusual result could have important implications for studies of early heart development in *Xenopus* and it deserves further attention. This is because the onset of MHCα expression in *Xenopus* embryos and more generally the beginning of cardiomyocyte differentiation are thought to occur after st. 28 (Logan and Mohun, 1993), and because animal caps expressing GATA4-GR are thought to faithfully reproduce normal cardiomyocyte differentiation (Latinkic et al., 2003).

Our own RT-PCR expression analysis, using sequence-verified amplicons derived from the 5', central and 3' regions of the MHCα transcript, confirms that MHCα is only expressed from the onset of cardiomyocyte differentiation (st.28). We also find, in contrast to Afouda et al, that GATA4-GR injected animal caps only express MHCα on schedule, i.e. at the time the gene is expressed in sibling control embryos (Figure A1). We therefore consider GATA4-GR expressing animal caps to be a valid model of cardiogenesis that recapitulates normal course of cardiomyocyte differentiation. The reasons for discrepancy between our results and those of Afouda et al are unclear at present, but are likely to include the details of experimental design. Irrespective of the causes of the differences between our results, we feel that it is important to clarify the utility of both the GATA4-GR expressing animal cap model of cardiogenesis and of MHCα as a specific marker of cardiomyocyte differentiation.

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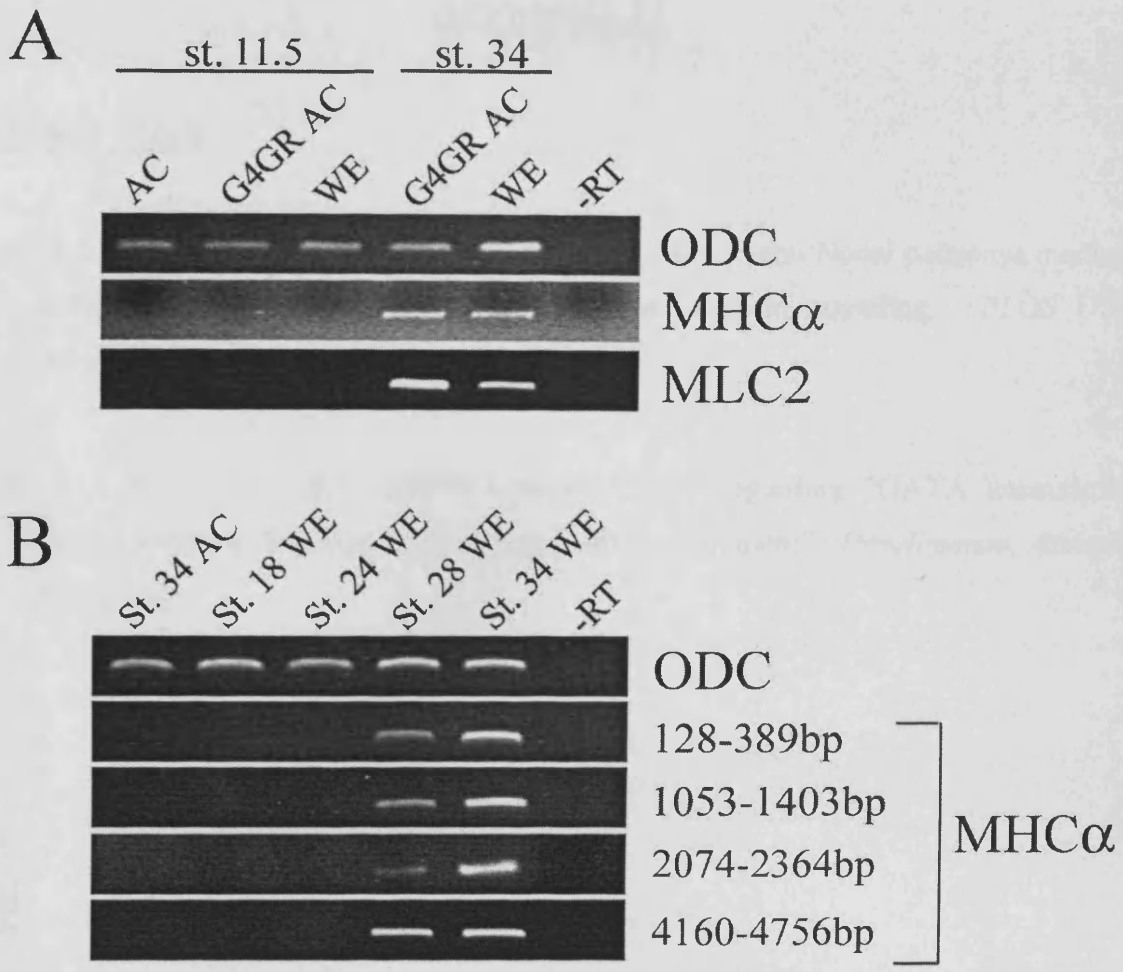

Figure A1 - Overexpression of GATA4 in animal caps recapitulates the normal course of cardiomyocyte differentiation

(A) GATA4-GR (G4GR), an inducible form of GATA4, induces in animal caps (AC) cardiomyocyte-specific markers MHCa and MLC2 at the time they are expressed in control sibling embryos, but not earlier (WE- whole embryos).

(B) MHCalpha expression, as assessed by RT-PCR using amplicons derived from the 5', central and 3' regions of the transcript, is initiated when cardiomyocyte differentiation is known to commence (around st. 28). ODC- loading control (ornithine decarboxylase).

APPENDIX II

Published Work

Samuel, L.J. & Latnkic, B.V. (2009) Early activation of FGF and Nodal pathways mediates cardiac specification independently of Wnt/β-catenin signaling. *PLOS ONE, **Accepted Manuscript***

Samuel, L.J. & Latinkic, B.V. (2009) Correspondence regarding "GATA transcription factors integrate Wnt signalling during heart development". *Development, **Accepted Manuscript***