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An investigation into a role for follistatin in early cardiogenesis in Xenopus Laevis

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An investigation into a role for follistatin in early cardiogenesis in *Xenopus Laevis*

(Spine Title: Investigations into Follistatin in Cardiogenesis)

By

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Abstract

Retinoic acid (RA) plays a crucial role in regulating cardiomyocyte differentiation and heart tube formation, but excess RA inhibits cardiomyocyte differentiation. We have used microarray technology and whole mount *in situ* hybridization to investigate the mechanism by which RA might alter cardiogenesis. Our results showed that RA inhibits cardiomyocyte differentiation at least partially through increasing follistatin gene expression and decreasing BMP signaling. Using cultured explants of the heart region, we demonstrated that addition of BMP4 and activin rescued the inhibitory effects of follistatin and RA on cardiomyocyte differentiation. These results indicate that the increased follistatin (FS) gene expression, known to decrease BMP signaling, may be responsible for the inhibition of heart development.

In order to investigate the role of follistatin in cardiogenesis, we also examined follistatin's developmental profile. We found that follistatin is expressed in the heart-forming region in early heart development, which suggests that follistatin is involved in cardiogenesis.

In addition, we first showed that excess RA inhibits *Tbx20* gene expression in the heart-forming region. This provides another mechanism by which excess RA inhibits cardiogenesis in addition to the known inhibition of *Nkx2.5* expression and BMP signaling.

Keywords: retinoic acid, cardiogenesis, *Xenopus,* follistatin, BMP, Nkx2.5.

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List of Abbreviations

°C	degree
%	percent
µg	microgram
µl	microliter
µM	micromolar
ActR-IA	activin receptor
ADH	alcohol dehydrogenase
AGN 194301	retinoic acid receptor alpha antagonist
ALDH	aldehyde dehydrogenase
ANF	Atrial Natriuretic Factor
AP axis	Anterior-Posterior axis
APC	Adenomatous Polyposis Coli
ATF	Activating Transcription Factor
AVC	atrioventricular canal
BMP	Bone Morphogenetic Protein
bp	base pair(s)
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CaMKII	Calcium/calmodulin dependent protein Kinase II
CBP	cyclic AMP-responsive element binding protein
cDNA	complementary deoxyribonucleic acid

CRABP	Cytoplasmic cellular Retinoic Acid Binding Protein
CO_2	carbon dioxide
cTnI	Cardiac Troponin I
CYP26	cytochrome P450, Family 26
dH_2O	distilled, autoclaved water
Dkk-1	dickkopf
DMSO	dimethylsulfoxide
DNAse	deoxyribonuclease
DBD	DNA-Binding Domain
Dpp	decapentaplegic
Dsh	Dishevelled
DTT	dithiothreitol
E	embryonic day
FGF	Fibroblast Growth Factor
FS	follistatin
FSH	Follicle stimulating hormone
FZ	frizzled
g	grams
GSK-3ß	Glycogen Synthase Kinase 3ß
HAT	histone acetyl transferase
hCG	human chorionic gonadotrophin
HDAC	histone deacetylase
HTLS	heat-treated lamb serum

LRP	Lipid Receptor Protein
IU	international units
IFT	inflow tract
JNK	c-Jun amino (N)-terminal Kinase
Nkx2.5	NK2 Transcription Factor related
LBD	Ligand-Binding Domain
LV	left ventricle
M	molar
MAB	Maleic Acid Buffer
Mef2	Myocyte Enhancer Factor 2
mg	milligram
ml	milliliter
min	minute
MLC	Myosin Light Chain
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
NCoR	Nuclear corepressor
NTP	nucleoside triphosphate
O_2	oxygen
O/N	overnight
OFT	outflow tract
PBS	phosphate buffered saline

PCP	Planar cell polarity
pH	potential of hydrogen
PKC	Protein Kinase C
RA	retinoic acid
RALDH	retinaldehyde dehydrogenases.
RAR (α/β/γ)	Retinoic Acid Receptor (alpha/beta/gamma subtype)
RARE	Retinoic Acid Response Elements
SMRT	Silencing Mediator of Retinoic acid receptor and Thyroid hormone receptor
St	embryonic stage
TAK	Transforming growth factor-ß Activated Kinase
TCF	T-Cell Transcription Factors
TGF-ß	Transforming Growth Factor-ß
VAD	vitamin A deficiency
WNT	wingless and int

CHAPTER 1 INTRODUCTION

1.1 Vertebrate cardiogenesis

1.1.1 Overview of heart development

In the earliest stages of development, the heart is a simple tube which consists of an epicardial layer, myocardial layer and endocardial layer. This three-layered tube is also subdivided into atria and ventricles. Heart development is initiated during late gastrulation in the anterior lateral plate mesoderm in *Xenopus* (Sater and Jacobson 1989). After the initial specification of cardiac precursors, the two bilaterally symmetric patches of cardiac progenitors extend laterally, eventually fuse and form the heart tube at the ventral midline of the embryo. The linear heart tube gradually bends and cardiac looping takes place to create an S-shaped loop (Chen et al., 1997).

As a result of cardiac looping, the posterior region of the heart tube lies dorsal relative to the more anterior, ventricular region and the entire heart tube is no longer linear along the anterior-posterior (AP) axis, rather it has formed an anticlockwise spiral. Finally, the looped heart tube must be further remodeled through continuing morphogenesis to form a mature heart.

1.1.2 Heart development in *Xenopus*

The adult form of *Xenopus* heart has septated atria resulting in separate pulmonary and systemic venous returns, a single atrio-ventricular valve, and a single, non-septated but highly trabeculated ventricle (Kolker et al., 2000; Mohun et al., 2000). Despite the anatomic differences in the mature hearts between *Xenopus* and the other

vertebrates, almost all of the known processes involved in early heart formation are conserved.

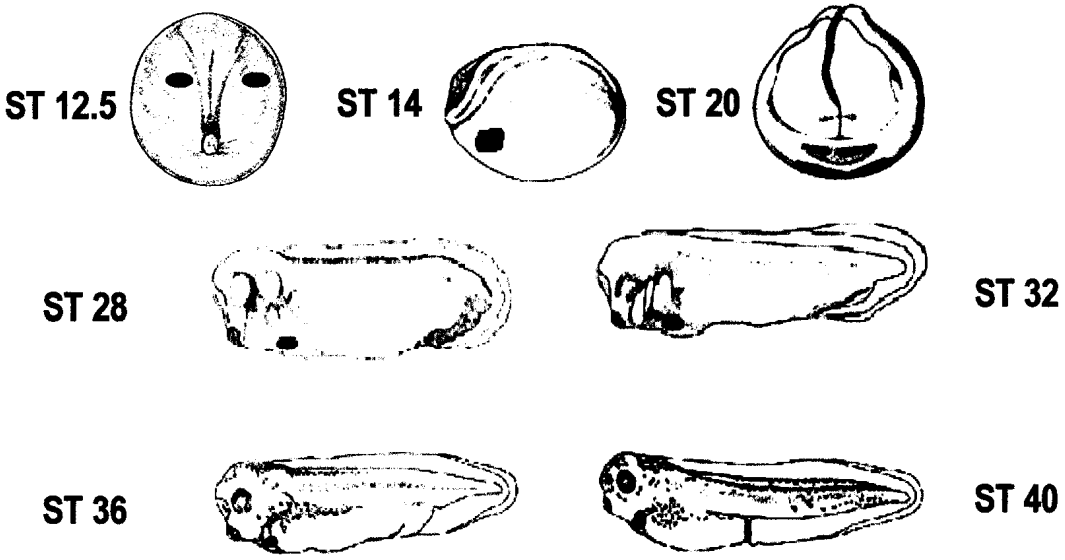
In *Xenopus*, as in all organisms, the heart arises from mesoderm. Mesoderm is the middle embryonic germ layer lying between the ectoderm and the endoderm from which connective tissue, muscle, bone, and the circulatory systems develop. Cardiac mesoderm is specified during gastrulation by signaling coming from the organizer and adjacent endoderm (Sater and Jacobson 1989). Through gastrulation movements, the cells of the cardiogenic mesoderm move forward to form a pair of the bilateral fields that migrate ventrally after gastrulation towards the ventral midline. By stage 13-15, *Nkx2.5* transcripts, the earliest marker of cardiogenic mesoderm, can be detected (Drysdale et al., 1997).

During the later tail bud stages, a single linear heart tube is formed with a prominent myocardial "trough" surrounding a thin endocardial tube (Mohun et al., 2000). This straight heart tube then undergoes dorsal and rightward looping, with the sinus venosus and atrial structures coming to lie anterior to the ventricle. After looping, the ventricular chamber thickens and becomes trabeculated. Fig.1.1 shows the locations of the heart in different stages of development in *Xenopus*.

1.1.3 Heart development in the mouse

In mouse, cardiac progenitor cells have been mapped to the anterior region of the primitive streak (Garcia-Martinez and Schoenwolf 1993). Two heart fields have been described in chick and mice. The first heart field derives from cells in the anterior lateral plate mesoderm that form a crescent shape at approximately embryonic (E) day 7.5 in the

Figure 1.1 The location of the heart in *Xenopus* in different stages. The region that is fated to become the heart is indicated in red. At stage 12.5, cardiac mesoderm is formed on the prospective dorsal side of the late gastrula, adjacent to the anterior axial somitic mesoderm. As a result of convergent extension movements, by the end of gastrulation at stage 14, the cardiac mesoderm lies at the dorsoanterior end of the embryo. During neurula stages at stage 20, the bilateral regions of cardiac mesoderm move ventrally and fuse on the ventral midline in the tailbud embryo. Successive stages of tadpole development (stages 28 and 32) with the prospective heart region are shown. At stage 36, cardiac looping is completed and a mature heart with chambered heart forms at stage 40.


ST 12.5
ST 14
ST 20
ST 28
ST 32
ST 36
ST 40

mouse embryo, corresponding roughly to week 2 of human gestation. By mouse E8.0, or 3 weeks in humans, these cells coalesce along the ventral midline to form a primitive heart tube, consisting of an interior layer of endocardial cells and an exterior layer of myocardial cells, separated by extracellular matrix (Srivastava 2006).

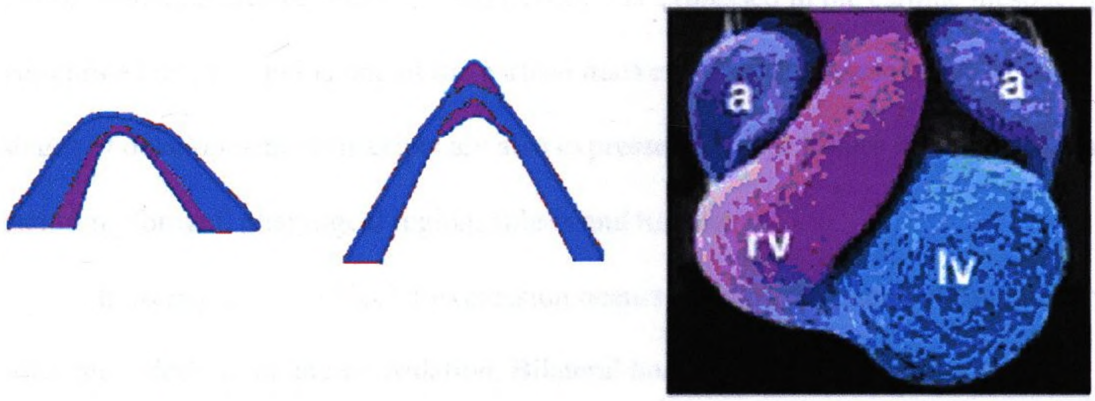
Based on fate-mapping, *in vivo* ablation experiments and gene expression studies, a novel heart-forming field, termed the second heart field, was identified (Mjaatvedt et al., 2001) (Fig.1.2). The secondary heart field comes from splanchnic mesoderm adjacent and caudal to the arterial pole of the heart (Waldo et al., 2001). The origin of this secondary field is mesoderm that lies near the heart-forming mesoderm very early in development and is displaced anteriorly. These distinct cells are marked by the expression of *fibroblast growth factor10* (FGF) and the *LIM-homeodomain transcription factor Islet1* (*Isl1*), and their fate is distinct from that of the rest of the heart (Cai et al., 2003). It is believed that the second heart field begins to appear after gastrulation and contributes to the outflow tract, right ventricle and aortic arch arteries (Srivastava, 2006).

As the cardiac tube grows, specified chambers begin to expand. The morphogenetic steps required to achieve looping are guided by molecular asymmetries established in and around the heart by the embryonic left/right axial pathway (Harvey 1998). Furthermore, in higher vertebrates, septal division of the chambers and formation of the valves are essential steps leading to the formation of an integrated four chambered heart with separate venous and arterial poles. During the growth process of the heart, another distinct cell lineage forms and develop into cardiac neural crest cells. These cells do not contribute to the heart directly. Instead, they migrate to the heart and are essential for the patterning of the great vessels and outflow septum (Harvey 1998; Zaffran and

Figure 1.2. Development of the embryonic mouse heart. At E 7.5, the anterior portions of the heart-forming fields have merged to form the cardiogenic crescent. The first and the secondary heart fields can be distinguished (respectively in blue and pink). At E 8.0, the linear heart tube has formed by progressive merging of the paired first and secondary heart fields. The first heart field coalesces along the ventral midline to form a primitive heart tube, consisting of an interior layer of endocardial cells and an exterior layer of myocardial cells, separated by extracellular matrix for reciprocal signaling between the two layers.

The first heart field gives rise to the left ventricle (lv) and portions of the atria (a), whereas the secondary heart field gives rise to the right ventricle (rv), portions of the atria, and the outflow tract. A scanning electron micrograph of a mouse heart at embryonic day 14.5 is shown with color coding to show late relative distribution of the two heart fields.

Derivatives of the first and secondary heart fields are shown in blue and pink, respectively. The atria, which are derived from the first and secondary heart fields, are shown in purple (adapted from Srivastava, 2006).



E 8 **8.5** **E 14.5**

Frasch 2002).

1.2 Cardiac transcription factors

1.2.1 Nkx2.5

Nkx2.5 is a member of the NK homeobox gene family and acts as a DNA-binding transcriptional activator (Harvey 2002). *Nkx2.5* is expressed in the cardiac mesoderm of vertebrate embryos and is one of the earliest markers of cardiac mesoderm. At later stages of development, transcripts are also expressed in other tissues during development, including foregut, pharyngeal region, spleen and tongue.

In *Xenopus*, early *Nkx2.5* expression occurs in both cardiogenic mesoderm and adjacent endoderm in late gastrulation. Bilateral heart primordia in anterior and lateroventral mesoderm at stage15 begin to fuse at the anterior midline between stages 16 and 19. This fusion is accompanied by a ventralward migration so that by stage 20, the single heart anlage is located ventrally, caudal to the cement gland. At these stages, *Nkx2.5* expression persists in both presumptive cardiac mesoderm and juxtaposed presumptive pharyngeal endoderm. Subsequently the *Nkx2.5*-positive endoderm migrates anteriorward relative to the cardiogenic mesoderm.

In *Xenopus* embryos, ectopic expression of *Nkx2.5* causes a modest increase in heart size (Cleaver et al., 1996). A similar increase in heart size has been reported in zebrafish embryos after *Nkx2.5* overexpression (Chen and Fishmen 1996) and forced expression of *Nkx2.5* in a zebrafish fibroblast cell line induces cardiac differentiation markers (Chen and Fishman 1996). In the mouse, targeted interruption of the *Nkx2.5* gene severely disrupts heart formation and the mice die around E9–10 due to the arrested looping morphogenesis of the heart tube and cardiac insufficiency (Lyons et al., 1995).

The expression of a dominant-negative mutant of *Nkx2.5* prevented the commitment of mesodermal cells into cardiac lineage during early P19 differentiation (Jamali et al., 2001). In human, heterozygous mutations in *Nkx2.5* have been identified to be causative for a variety of congenital heart diseases (Schott et al., 1998; Srivastava and Olson, 2000).

Nkx2.5 plays a crucial role in the transcriptional regulation of several sets of cardiac-specific genes (Akazawa and Komuro 2005). Nkx2.5 strongly activates the atrial naturetic peptide (*ANP*) promoter and the activation of the *ANP* promoter is dependent on the Nkx2.5 DNA-binding site located within the *ANP* promoter. In addition to *ANP*, other direct downstream targets for Nkx2.5 have been identified. These target genes encode important structural proteins and transcriptional regulators that confer features characteristic of cardiomyocytes, supporting a functional role of *Nkx2.5* in the transcriptional regulation of a cardiac gene program (Akazawa and Komuro 2005).

1.2.2 GATA4, 5 and 6

The GATA proteins have been divided into two subfamilies, GATA1, 2, 3 and GATA4, 5, 6. *GATA1, 2,* and *3* genes are prominently expressed in hematopoietic cells and some ectodermal derivatives, while *GATA4, 5,* and *6* genes are expressed in various mesoderm- and endoderm-derived tissues such as heart, liver, lung, gonad, and gut where they play critical roles in regulating tissue-specific gene expression. In *Drosophila,* a single *GATA* gene, *pannier,* is required for cardiogenesis to occur (Gajewski et al., 1999). *Pannier* functions as a cardiac identity gene as forced expression results in increased number of cardiac cells at the expense of other derivatives of the dorsal mesoderm. Pannier works synergistically with tinman, a homologue of *Nkx2.5* in *Drosophila* , and

ectopic coexpression of both factors results in expanded cardiogenic gene expression domains in ventral and dorsal mesoderm (Gajewski et al., 1999).

Mouse embryos that lack *GATA4* have bilateral heart tubes (*cardia bifida*) and a reduced number of cardiac myocytes (Kuo et al., 1997). Overexpression of *GATA4* in embryonic stem cells displays enhanced cardiac differentiation and depletion of *GATA4* prevented terminal differentiation into cardiomyocytes. Gain-of-function experiments caused premature expression of cardiac differentiation markers and in presumptive ectoderm induced heart marker expression and in some cases spontaneous cardiomyocyte beating in *Xenopus* embryos.

GATA4 regulates expression of a number of cardiac structural genes and also regulates developmental expression of the cardiac transcription factor Nkx2.5, suggesting the existence of a reinforcing transcriptional regulatory circuit between Nkx2.5 and GATA factors in the heart (Chen et al., 1996). GATA4 physically interacts with muscle enhance factor in the regulation of cardiac gene expression.

Null mutations in *GATA5* caused cardiac defects in zebrafish and moreover, the expression of the early regulator *Nkx2.5* was also down regulated. *GATA5* knockout mice are viable and fertile, but females exhibited pronounced urogenital defects. Overexpression of *GATA5* in zebrafish embryos was not only able to increase the expression of *Nkx2.5*, *GATA4*, *6* and later myocardial genes in cardiac tissue, but also induced ectopic expression of some of these genes in non-cardiac tissue. Inhibition of GATA5 in these cell lines blocked terminal differentiation at a pre-endocardial stage. This observation is consistent with the expression pattern of *GATA5* which becomes restricted to the endocardium during later development.

Gain-of-function experiments in *Xenopus* embryos showed that *GATA6* plays a role in cardiogenesis. Elevating *GATA6* delayed the onset of terminal differentiation of cardiomyocytes. These results indicated that *GATA6* is involved in cardiogenesis.

1.2.3 T-Box genes

The T-box gene family is defined by a common DNA binding domain known as the T-box and these genes play diverse roles in embryonic development. The T-box encodes a DNA-binding domain of 180 amino acid residues. In human, both spontaneous and induced mutations in T-box genes have demonstrated that they are major contributors to several human syndromes.

Many T-box genes are expressed in specific chambers or regions of the developing vertebrate heart, including *Tbx1*, *Tbx2*, *Tbx3*, *Tbx5*, *Tbx18*, and *Tbx20.* Targeted mutagenesis in mouse has revealed essential and unique roles for *Tbx1*, *Tbx2*, *Tbx5*, and *Tbx20* in cardiac development.

Homozygous loss of *Tbx1* produces abnormalities of anterior cardiac development, including shortening of the outflow tract, the absence of outflow tract (OFT) septation, ventricular septal defects, and abnormal remodeling of the aortic arch arteries. Conversely, ectopic expression of *Tbx1* in the heart tube can lead to an elongated OFT. *Tbx1* mutants display reduced proliferation in the anterior heart field, which contributes to the OFT (Xu et al., 2004), demonstrating a role in proliferation of cardiac progenitor cells.

Embryos homozygous for a null mutation in *Tbx2* reveal a role for the gene in repressing chamber differentiation in the atrioventricular canal (AVC) during functional

specialization of the ventricular and atrial compartments. Many *Tbx2* homozygous null mutants also exhibit defects in OFT septation and remodeling of the aortic arch arteries.

Tbx5 expression is maintained in the structures derived from the posterior domains of the linear heart tube, the atria and left ventricle. Mice lacking *Tbx5* show severe hypomorphic development of the developing atria in the primitive heart tube and do not survive beyond embryonic day (E) 10.5 (Bruneau et al., 2001). Conversely, transgenic overexpression of *Tbx5* inhibits ventricular chamber maturation and ventricle-specific gene expression, which indicates that *Tbx5* plays a role in the determination of atrial vs ventricular formation (Liberatore et al., 2001). Tbx5 is also essential for later cardiac events such as development of conductive system and position of the septum (Bruneau et al., 2001).

Tbx5 expression in the primitive heart is dependent on retinoic acid signaling in both chicken and mouse embryos (Liberatore et al., 2000; Niederreither et al., 2001). In retinoic acid-treated chicken embryos, the atrial expression of *Tbx5* is expanded anteriorly (Liberatore et al., 2000). In addition, mice lacking the retinoic metabolic enzyme RALDH2 have impaired atrial development and reduced *Tbx5* expression (Niederreither et al., 2001). These embryonic studies support a critical role for *Tbx5* downstream of retinoic acid signaling in atrial lineage specification and development of the posterior segments of the primitive heart tube.

1.2.4 Tbx20

Tbx20 is a T-box transcription factor that is expressed throughout the early cardiac crescent, and later in both myocardium and endocardium. In zebrafish and

Xenopus, loss or gain-of-function of *Tbx20* causes abnormal cardiogenesis (Brown et al., 2005).

Tbx20 is expressed in the primitive heart tube, atria, and developing valves in mice (Cai et al., 2005). Initially *Tbx20* expression was reported in the lateral plate mesoderm, heart primordia, primitive heart tube, and four-chambered heart of mouse and chicken embryos. Subsequent studies have shown enrichment of Tbx20 in the atria, outflow tract, and valve primordia of the heart.

Expression of *Tbx20* overlaps with *Tbx5* in the atria and with *Tbx2, Tbx3,* and *Tbx5* in the AV canal myocardium, suggesting coordinate regulation of T-box target gene expression in these structures. Tbx20 synergizes with GATA4 to activate the *Mef2c* enhancer and *Nkx2.5* enhancer.

Homozygous null *Tbx20* mutants die midgestation due to defective hearts, which fail to loop and which display many morphological and molecular abnormalities, including widespread up-regulation of *Tbx2*. The presence of two poorly developed chamber-like structures suggests that *Tbx20* plays a role during cardiac chamber formation in the early heart tube (Cai et al., 2005).

1.3 Signaling events in cardiogenesis

Cardiogenesis is a complex process which involves multiple signaling pathways. At present, four classes of signaling factors have been characterized extensively for their roles during the early heart development. These signaling molecules are Wnts, bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and retinoic acid (RA). They cooperate in the regulation of cardiogenesis.

1.3.1 Wnt signaling pathways

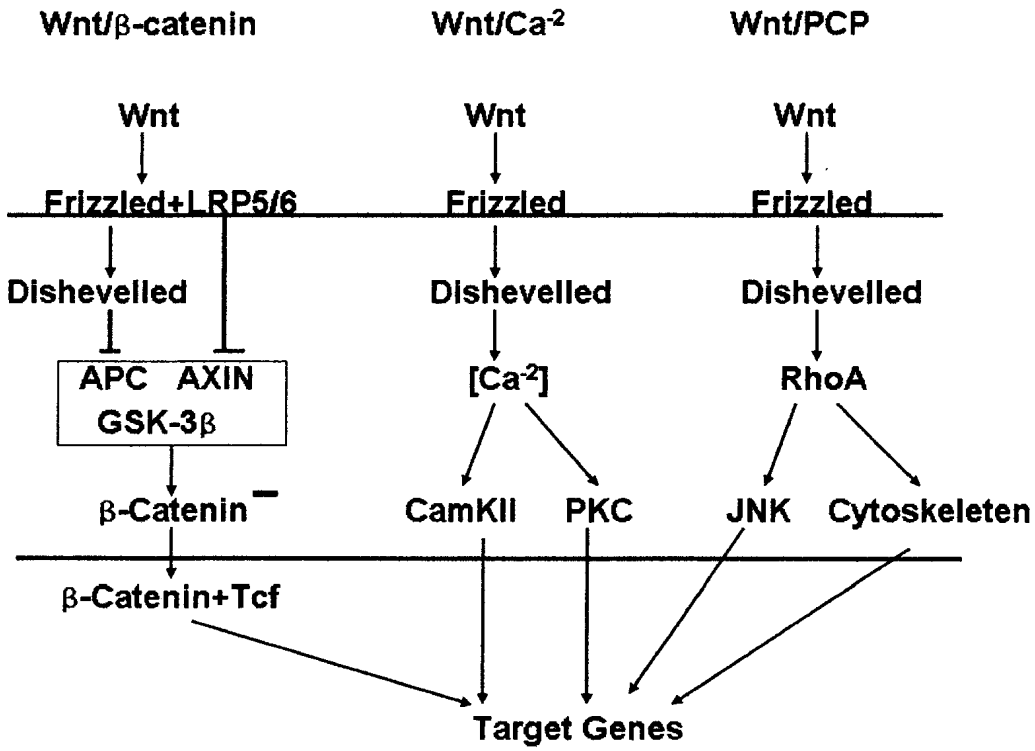
Wnts are a family of secreted protein with *wingless* as the initial member discovered in the family. *Wnt* family genes encode secreted proteins that can function at a distance from their source and can activate different intracellular signaling pathways through interactions with their transmembrane receptor frizzled and co-receptor LRP-5/6 (Fig. 1.3).

After the wnt ligand binds frizzled, the Wnt signaling pathway diverges into three branches: the Wnt/β-catenin pathway (canonical pathway), the planar cell polarity (PCP) pathway and the Wnt/calcium pathway. In the canonical pathway, activated disheveled (Dsh) suppresses phosphorylation of glycogen synthase kinase 3β (GSK-3β), leading to its polyubiquitination and degradation by the 26S proteasome (Aberle *et al.,* 1997; Peifer *et al.,* 1994; Rubinfeld *et al.,* 1996). In the absence of Wnt ligand, the destruction complex, which consists of the scaffolding proteins APC, Axin and the serine/threonine kinase GSK-3β, targets β-catenin to the ubiquitin- proteasome complex for degradation. Binding of Wnt to receptors results in stabilization of β-catenin, which translocates to the nucleus where it binds to transcription factors (TCF) and co-activators (CBP and p300) to activate a transcriptional program (Kohn et al., 2005).

The PCP pathway controls the polarity of cells during gastrulation (Heisenberg et al., 2000). In *D. melanogaster*, activated frizzled (FZ) signals to Dsh, which activates the small GTPase RhoA and c-Jun amino (N)-terminal kinase (JNK). In the Wnt/calcium pathway, binding of specific Wnts to FZ receptors leads to signaling through Dsh to induce calcium influx and activation of protein kinase C (PKC) and calcium/calmodulin dependent protein kinase II (CaMKII).

Figure 1.3. Wnt signaling pathways. Wnt proteins bind to the Frizzled/LRP receptor complex at the cell surface. These receptors transduce a signal to Dishevelled (Dsh) and to Axin, which may directly interact. As a consequence, the degradation of *β*-catenin is inhibited, and this protein accumulates in the cytoplasm and begins to move into the nucleus where β-catenin can then interact with TCF to control transcription of taget genes. In the Wnt-calcium pathway. binding of Wnt to Frizzled leads to activation of Dsh and an increase in intracellular calcium and activation of PKC as well as activation of calmodulin kinase II (CamKII). Each of these factors can then mediate various intracellular responses. In the Wnt-PCP pathway. activated Frizzled receptors again lead to activation of Dsh, which then signals through small GTPases and C-Jun N-terminal kinase to modulate cytoskeletal elements including actin and microtubules.

TCF, T-Cell transcription factors; PKC, protein kinase C; CaMKII, calcium/calmodulin dependent protein kinase II; JNK, c-Jun amino (N)-terminal kinase; PCP, planar cell polarity; GSK3β, glycogen synthase kinase 3β (adapted from Kohn et al., 2005).


Wnt/β-catenin
Wnt/Ca-2
Wnt/PCP
Wnt
Wnt
Wnt
Frizzled+LRP5/6
Frizzled
Frizzled
Dishevelled
Dishevelled
Dishevelled
APC AXIN
GSK-3β
[Ca-2]
RhoA
β-Catenin
CamKII
PKC
JNK
Cytoskeleten
β-Catenin+Tcf
Target Genes

In chicken and *Xenopus*, activation of the canonical Wnt signaling pathway inhibits cardiomyocyte differentiation. Overexpression of Wnt3A and Wnt8 blocks expression of *Nkx2.5*, *Tbx5*, and cardiac differentiation markers (Schneider et al., 2001). However, Wnt11 (Wnt5 class) stimulates heart muscle cell formation by signaling through the planar polarity (JNK) pathway and inhibiting the canonical pathway (Pandur et al., 2002). In *Xenopus*, *Wnt11* is expressed in the anterior lateral mesoderm and interference with Wnt11 signaling results in loss of heart muscle cell formation. Wnt11 can also induce heart muscle cell formation in mouse embryonic carcinoma (P19) cells (Pandur et al., 2002). Wnt antagonists such as crescent and Dkk-1 are able to initiate cardiogenesis in non-cardiogenic tissues. They are the secreted factors, which bind and inhibit specific Wnt ligands, including Wnt3A and Wnt8. When mesodermal cells migrate anteriorly, Wnt signaling is inhibited by Crescent (Bodmer and Frasch 1999) and/or Dkk-1, relieving the block on the initiation of the cardiac gene expression program. Wnt antagonists function to reverse the repression in cardiac induction in both chick and frog (Schneider and Mercola 2001). Wnt antagonists are expressed throughout the entire anterior portion of the embryos during early heart development, but the cardiac gene expression is only initiated in the anterolateral heart-forming regions (Olson et al., 2005). This implies that additional factors are needed for proper induction of the cardiac gene expression. Figure 1.4 shows a proposed model of how Wnt signaling initiates cardiogenesis.

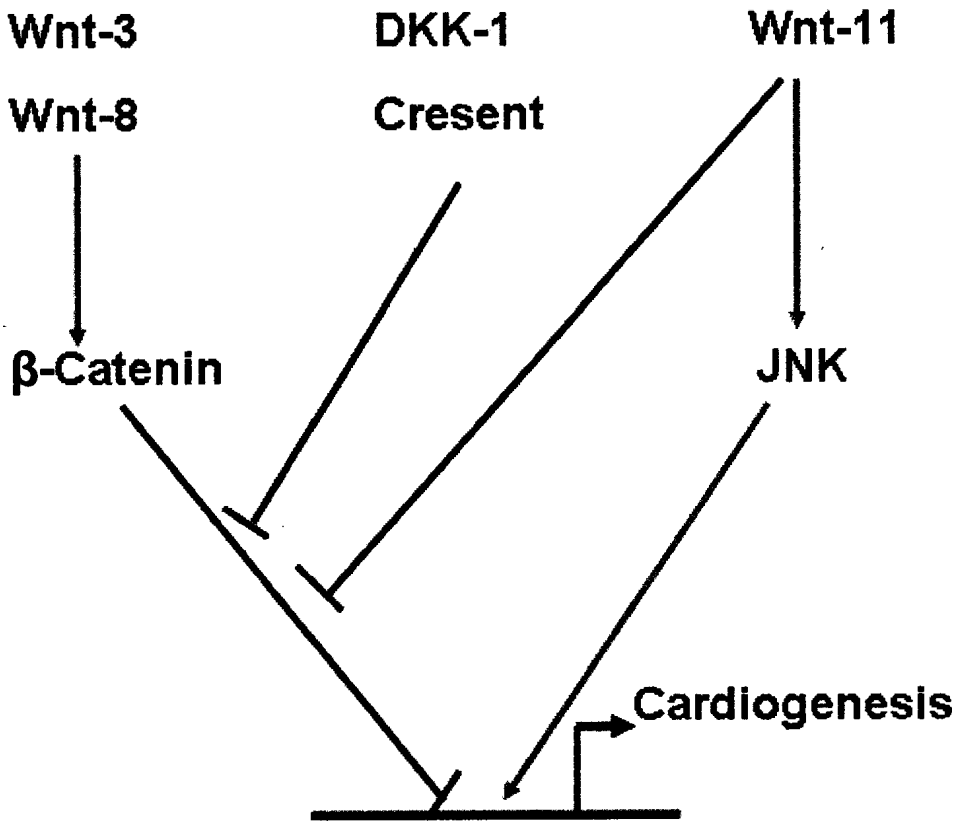
1.3.2 Bone morphogenetic protein signaling pathways in cardiogenesis

Bone morphogenetic proteins (BMPs) are secreted signaling molecules

belonging to the transforming growth factor-β (TGF-β) superfamily of growth factors. The first BMPs were originally identified by their ability to induce ectopic bone formation when implanted under the skin of rodents. To date, around 20 BMP family members have been identified and characterized. BMPs play important and diverse roles in the regulation of embryonic development and cellular functions. BMPs signal through specific serine/threonine kinase receptors. There are three kinds of BMP receptors which bind BMP ligands: BMPR-IA, BMPR-IB and type IA activin receptor (Chen., 2004). Signal transduction studies have revealed that Smad1, 5 and 8 are the immediate downstream molecules of BMP receptors and play a central role in BMP signal transduction. Smads1, 5 and 8 transiently and directly interact with activated type I BMP receptors, which phosphorylate Smad in a ligand-dependent manner (Chen et al., 1997). After release from the receptor, the Smad1, 5 and 8 form complex with Smad4. This complex translocates into the nucleus and participates in gene transcription with other transcription factors.

Evidence for a direct involvement of BMP signaling in early cardiogenesis was initially obtained in the *Drosophila* system through studies of decapentaplegic (Dpp), the *Drosophila* homologue of BMP. During gastrulation and mesoderm migration, Dpp is expressed in a broad band of cells within the dorsal ectoderm (Chen and Fishman 1996). Embryos that are mutant for *Dpp* or other components of the Dpp pathway lack dorsal mesodermal expression of *tinman*, a homedox-containing gene necessary for specification of the heart. Ectopic activation of the Dpp pathway in the ventral-most areas of the mesoderm ultimately leads to ectopic formation of dorsal vessel (heart) cells (Yin et al., 1998).

Figure 1.4. The effects of Wnt signaling molecules on cardiogenesis. Wnt -3A and Wnt-8 inhibit heart development through β-catenin. Wnt inhibitors such as crescent and DKK-1 antagonize Wnt signaling and initiate cardiac development. Alternatively, Wnt-11 can induce heart development through activating JNK pathway and inhibiting Wnt-3A and 8 signaling (adapted from Pandur et al., 2002).


Wnt-3
Wnt-8
DKK-1
Cresent
Wnt-11
β-Catenin
JNK
Cardiogenesis

BMPs are expressed in the region of the precardiac mesoderm in vertebrates. *BMP2* is expressed in the endoderm and *BMP4* and *BMP7* are expressed in the ectoderm overlaying the precardiac mesoderm in chick and *Xenopus* (Schultheiss et al., 1997; Shi et al., 2000). Application of BMP to the heart-forming regions results in an expansion of the *Nkx2.5* expression domain (Andree et al., 1998). In the chick, explants of non-cardiac (anterior medial) mesoderm from the mid/late gastrula embryo have been induced to undergo terminal cardiac differentiation by exposure to BMP2 and BMP4 (Schultheiss et al., 1997). These results suggest that BMPs act as positive regulators of cardiac fate in vertebrates as well as *Drosophila*.

The requirement for BMP signaling in cardiogenesis has been further confirmed by studies using inhibitors of BMP signaling, including noggin, truncated versions of type I or II BMP receptors, and inhibitory Smad6 (Shi et al., 2000; Walters et al., 2001). When these inhibitors of BMP signaling are introduced into the organisms, there is a block to heart differentiation (Shi et al., 2000). BMP signaling is required for heart formation, expression of differentiated markers, and complete fusion of heart primordia. Whole-mount *in situ* analysis indicates that ectopic expression of dominant negative BMPRII resulted in a significant number of mutant embryos with reduced or abrogated expression of two markers of the differentiated heart, *myosin light chain* (*MLC2*) and *cardiac troponin I* (*cTnI*) in *Xenopus* (Shi et al., 2001).

TGF-β activated kinase 1 (TAK1) is a member of the MAPKKK superfamily (Yamaguchi et al., 1995). TAK1 is activated by BMPs and overexpression of wild-type TAK1 induces ventralization of *Xenopus* embryos, while kinase-negative TAK1 inhibits constitutively active BMP receptor-induced ventralization. Overexpression of TAK1

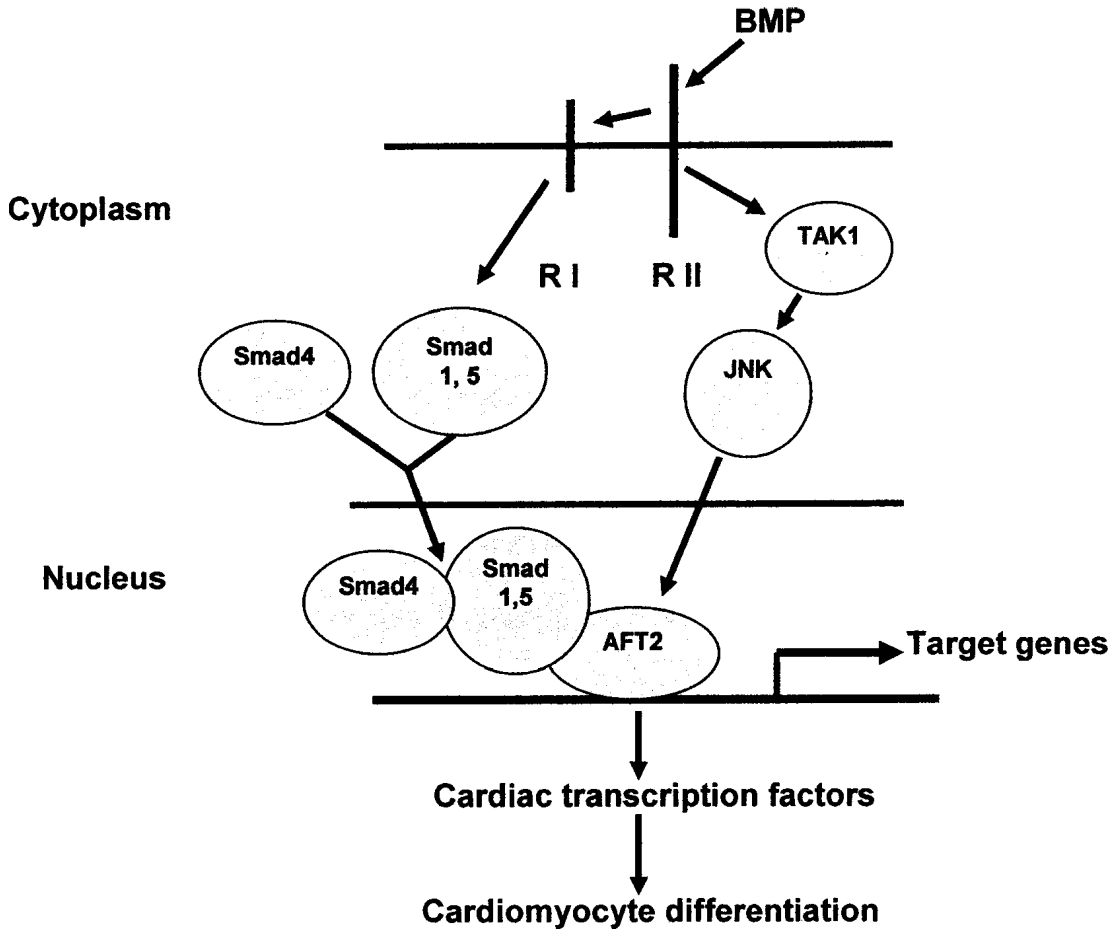
restored the ability of P19CL6-noggin cells to differentiate into beating cardiomyocytes, whereas overexpression of the dominant negative form of TAK1 in parental P19CL6 cells reduced the differentiation efficiency into cardiomyocytes, suggesting that the TAK1-mediated MAPK pathway was involved in BMP-induced differentiation of cardiac precursor cells. Figure 1.5 summarizes the role of BMP signaling in cardiomyocyte differentiation.

BMPs induce expression of some cardiac-specific genes and terminal cardiomyocyte differentiation through the Smad and the TAK1 pathways (Monzen et al., 2001). Both pathways cooperatively activate some sets of genes related to terminal cardiomyocyte differentiation. BMP signal transduction pathways and its downstream transcription factors are the central molecules of this regulatory network controlling cardiac differentiation. For example, Nkx2.5 is one of important cardiac transcription factors and BMP signaling is required to maintain *Nkx2.5* expression prior to differentiation (Shi et al., 2001).

1.3.3 Fibroblast growth factor signaling pathway

Fibroblast growth factors (FGFs) represent a large family of secreted molecules: ten members have been identified in zebrafish, six in *Xenopus*, thirteen in chicken and twenty-two in mouse and human. FGFs act through binding and activation of FGF receptors that are transmembrane proteins with tyrosine kinase activity. Upon binding to their cognate receptors, FGFs activate signal transduction pathways which are required for multiple developmental processes. All FGFs share a high affinity for heparin. FGF receptor transmits extracellular signals to various cytoplasmic signal transduction

Figure 1.5. BMP signaling in cardiomyocyte differentiation. BMP dimers bind to serine/threonine kinase receptors type I and II. Upon ligand binding, type II receptors phosphorylate type I receptors. The latter phosphorylates Smad1 and Smad5 which form a complex with Smad4. These Smads are subsequently translocated to the nucleus, where they activate transcription of target genes. In addition, receptor II also can activate TAK1 which activates target genes through JNK. ATF, activated factor; TAK1, TGF-β activating kinase (adapted from Monzen et al., 2001).


BMP
Cytoplasm
TAK1
R I
R II
Smad4
Smad
1, 5
JNK
Nucleus
Smad4
Smad
1,5
AFT2
Target genes
Cardiac transcription factors
Cardiomyocyte differentiation

pathways through several pathways.

There is substantial evidence from both *Drosophila* and vertebrates that FGF signaling makes a direct contribution to the specification of heart progenitors. In the chick, FGF2 and 4 can induce cardiogenesis in non-precardiac mesoderm (Barron et al., 2001). FGF8 is expressed in the endoderm adjacent to the precardiac mesoderm and previous studies have found that this endoderm plays an important role in heart specification. Removal of the endoderm results in a rapid down-regulation of a subset of cardiac markers, including Nkx2.5 and muscle enhance factor (Mef2c). Expression of these markers can be rescued by supplying exogenous FGF8 to the embryos when the endoderm has been removed. In addition, application of ectopic FGF8 results in ectopic expression of cardiac markers. However, the expression of cardiac markers is expanded only in regions where BMP signaling is also present (Barron et al., 2001).

Zebrafish FGF8 is expressed in the cardiogenic fields of the lateral plate and in specific areas of the neural tube and *fgf8* (*acerebellar*) mutant embryos fail to initiate proper expression of the cardiac transcription factors Nkx2.5 and GATA4, resulting in a severely malformed heart with reduced ventricle (Reifers et al., 2000). In addition, FGF receptor dominant-negative and antisense constructs injected into embryonic day 3 heart suppressed cardiomyocyte proliferation during the first week of embryogenesis in chick.

1.3.4 Follistatin

Follistatin (FS) is a secreted protein, which was originally isolated from porcine follicular fluid as an inhibitor of follicle stimulating hormone (FSH) secretion from pituitary cells (Esch et al., 1987). Later, it was found that FS acted by antagonizing

activins and BMPs (Phillips et al., 1998). The affinity of FS for activin is very high, with Kd estimates ranging from 50 to 900 pM suggesting that the binding of activin to FS is almost irreversible (Patel 1998). FS antagonizes the action of activin by forming a FS-activin complex that is rapidly destroyed by lysosomal enzymes after binding to cell surface proteoglycans and being internalized (Patel 1998). FS can also interact with BMPs and facilitate clearance of BMPs in a similar manner as it does activin (Patel 1998). FS exists in many tissues where activins and BMPs are made (Patel 1998). Figure 1.6 summarizes the interactions between follistatin and activin or BMPs at cell surface.

FS first appears at the dorsal lip of the blastpore in *Xenopus* (Hemmati-Brivanlou et al., 1994). FS expression is maintained in the prechordal mesoderm and anterior two thirds of the notochord in late gastrula stage embryos. In early neurulae stage embyros, the expression of follistatin is confined to head mesoderm and anterior notochord. As the development proceeds, follistatin begins to appear in the brain, kidney and blood islands.

In mice, FS knockout animals die within the first few hours after birth because they fail to breathe, which prevents the assessment of the physiological roles of FS in the neonate and adult (Matzuk et al., 1995). These mice display skeletal abnormalities and less muscle in their diaphragm (Matzuk et al., 1995). Overexpression of follistatin in the skeletal muscle of mice results in an increase in muscle mass (Hill et al., 2000). Noggins, chordin and follistatin are all known BMP inhibitors and play a crucial role in the induction of neural development.

Recently, a knockdown of three BMP antagonists, FS, noggin and chordin, in the organizer region demonstrate that there is an essential and cooperative role for these antagonists in normal dorsal axis development (Khokha et al., 2005). Embryos that

cannot effectively reduce the BMP signal on their dorsal side develop excessive amounts of ventral tissue at the expense of dorsal structures. Knockdown of any two of them has mild effects, but the neural plate still forms. However, once all three BMP antagonists are inhibited, a catastrophic failure of dorsal development occurs and the ventral and posterior fates expand. This suggests that these three BMP antagonists have overlapping and cooperative functions that are critical for dorsal development.

Follistatin is able to block the mesoderm-inducing activity of activin (Asashima et al., 1992), BMP4 (Fainsod et al., 1997), and BMP7 (Yamashita et al., 1995). Follistatin is believed to block the effects of BMP in concert with the other BMP antagonists such as noggin and chordin (Melton et al., 1997). Gain of function for follistatin's role showed that injection of follistatin RNA into *Xenopus* animal cap explants resulted in the induction of neural tissue with specific neural markers and enlarged notochordal tissue. which indicates that follistatin can induce neural development by opposing BMP signaling.

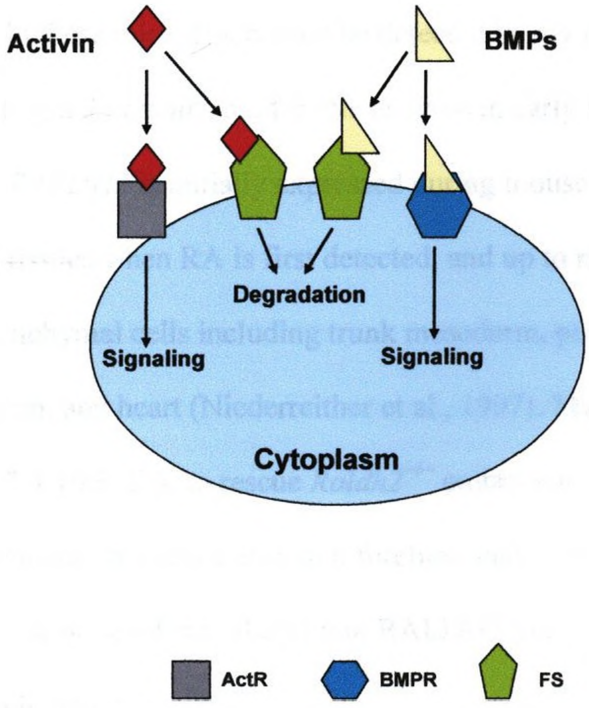
To date, the role of follistatin has not been understood in cardiac development. This is one of the objectives of this thesis.

1.3.5 Retinoic Acid

1.3.5.1 RA synthesis and degradation

Retinoic acid (RA) is expressed in the embryo and plays an important role in regulating metabolism, growth, differentiation and development. The enzymes responsible for RA synthesis play a key role in controlling the level of RA. The major RA synthesis enzymes are retinol dehydrogenases and retinaldehyde dehydrogenases

Figure 1.6. Interactions between follistatin and activin or BMPs. A clearance model of activin or BMPs by FS is shown. In this model, if circulating activin (Act) or BMP bind to their receptors (ActR or BMPR), they can induce their signaling pathways. Once they are captured by surface bound follistatin, the follistatin/activin complex or follistatin/BMP complex is internalized. Lysozymes within the cell are responsible for breakdown of the complexes. Follistatin is associated with heparan sulphate proteoglycans and exists at the cell surface (adapted from Patel 1998).


Activin
BMPs
Degradation
Signaling
Signaling
Cytoplasm
ActR
BMPR
FS

(RALDH). The former is responsible for the efficient conversion of retinol to retinaldehyde and the latter for retinaldehyde to RA (Fig. 1.7).

Among the enzymes responsible for RA synthesis, RALDH2 is the rate-limiting enzyme that is responsible for almost 90% of RA production. *RALDH2* knockout mice die at mid-gestation due to severe trunk, hindbrain and heart defects (Niederreither et al., 1999). In these mice, RA cannot be detected in any tissue except for the developing eye, supporting a dominant role for this enzyme in early RA synthesis.

RALDH2 is initially expressed during mouse embryogenesis at E7.5 primarily in cranial tissues when RA is first detected, and up to midgestation it is localized primarily in mesenchymal cells including trunk mesoderm, proximal limb bud and lung bud mesoderm, and heart (Niederreither et al., 1997). Maternal retinoic acid administration from E7–E10 is able to rescue *Raldh2*$^{-/-}$ embryonic development to the near full rescue of the mutant phenotype although forelimb buds are truncated (Niederreither et al., 1999). The knock-out evidence shows that RALDH2 plays a fundamental role in providing RA for development.

In *Xenopus,* localized expression of *RALDH2* can first be detected in gastrulation in the mesoderm around the vegetal pole and this expression is more intense in the region of dorsal blastpore lip (Chen, 2001). As gastrulation proceeds, *RALDH2* expressing cells involute into the trunk region of the embryo at the dorsal and lateral sides. At the end of gastrulation, the signal becomes restricted to the middle part of the trunk flanking the notochord. During neurula stages, the trunk mesodermal RALDH2 positive domains expand ventrally and meet at the ventral midline (Chen, 2001). RA produced in tissues is committed either to activating receptors to regulate RA-responsive genes or to being

degraded to inactive forms by the RA metabolic machinery.

The metabolism of RA is initiated by hydroxylation and degraded by the enzyme CYP26. CYP26 metabolizes all *trans* RA but does not metabolize the 9-*cis* or 13-*cis*-RA isomers (Ross 2003). The inducible expression of *CYP26* in mouse embryos also suggests that a similar auto-regulatory mechanism may limit exposure to RA sensitive tissues during development (Iulianella et al., 1992). *CYP26* knockout mice die before birth and displays a spectrum of severe abnormalities resulting from excessive RA, including exencephaly, spina bifida, posterior body truncations and hindlimb fusion (Ross 2003).

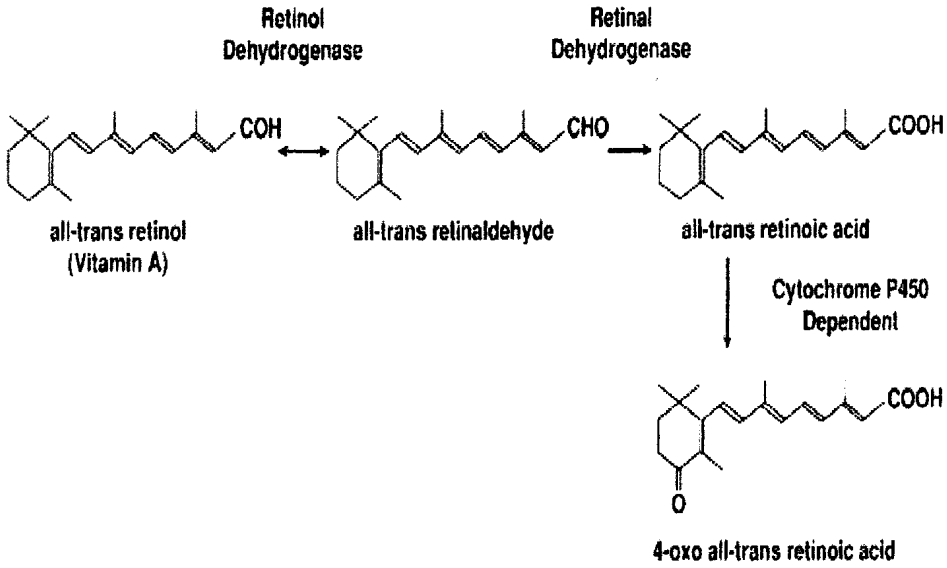
In *Xenopus laevis,* whole-mount *in situ* hybridization revealed *CYP26* transcripts in the mesoderm around the future blastopore at the onset of gastrulation (stage 10). In early gastrula (stage 10.5), this expression ring intensifies and staining is also observed in the dorso-anterior region of frogs. By late gastrula (stage 12.5), a posterior-expression domain and a dorso-anterior expression domain become well defined. By neurula stage 17 there is still strong expression around the blastopore. More anteriorly, expression is observed in the cement gland, at the lateral border of the anterior neural plate, and at the edge of the neural fold at the level of the future hindbrain (Ross 2003).

By later tailbud stages (stage 32), *CYP26* is still expressed in the tip of the tail, the eye cup, and the lens anlage. The *CYP26* expression suggests that maintenance of suitable levels of RA by CYP26 is important for tissue or organ development.

1.3.5.2 RA receptors

In the nucleus, there are two families of retinoid nuclear receptors involved in

Figure 1.7. The metabolic flow from retinol to retinoic acid and its oxidation. Inside the cell, the sequestered retinol is enzymatically converted first to retinal by the retinol dehydrogenases (ADHs) and then to RA by the retinal dehydrogenases (RALDHs). There are three members of the RALDH family. Among all the enzymes implicated in RA synthesis, RALDH2 is a key enzyme in the localized production of RA. RA is further metabolized by a cytochrome P450 enzyme called CYP26 to inactive products and finally discharged (Deluca et al., 2002).

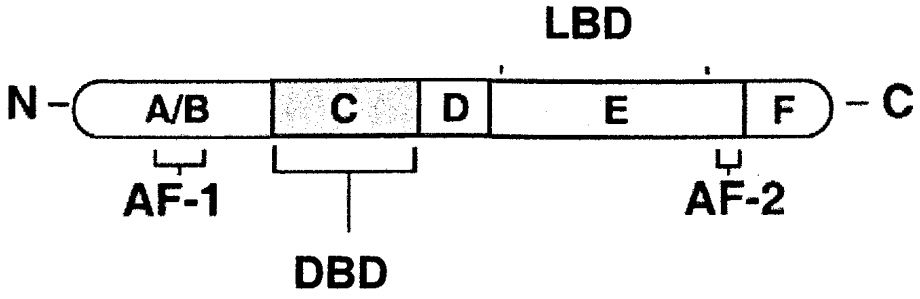

Retinol
Dehydrogenase
Retinal
Dehydrogenase
COH
CHO
COOH
all-trans retinol
(Vitamin A)
all-trans retinaldehyde
all-trans retinoic acid
Cytochrome P450
Dependent
COOH
O
4-oxo all-trans retinoic acid

the regulation of the effects of RA on gene expression. They are RA receptors (RARs) and retinoid X receptors (RXRs). Each of them is comprised of three subtypes: α, β, and γ encoded by different genes (Chambon 1996) and RARs and RXRs form heterodimers. Most tissues, especially during embryonic development, express one or more RAR and RXR subtypes in different combinations, possibly giving rise to different responses to RA in different tissues. The distribution of RA is also an important determinant in regulating RA responsive genes, especially in the development of tissues.

Like most nuclear hormone receptors, retinoid receptors exhibit a modular structure (Fig. 1. 8). The highly conserved C region harbors the DNA-binding domain (DBD) which confers sequence specific DNA recognition. Region E is the second most conserved region and corresponds to the ligand-binding domain (LBD). The N-terminal A/B region harbors a ligand-independent transcriptional activation function (AF-1). The D region serves as a hinge between the DBD and the LBD, allowing rotation of the DBD. Therefore, it might allow the DBD and the LBD to adopt different conformations. It also harbors nuclear localization signals (Marill et al. 2003).

RARα, RAR β and *RAR*γ-null mutant mice are viable due to the existence of functional redundancies between RARs (Ghyselinck et al., 1997; Luo et al., 2003). However, they display some aspects of the fetal and postnatal vitamin deficiency disease (VAD) syndromes. Their abnormalities are restricted to a subset of tissues normally expressing these receptors. *RAR* α/β, *RAR* α/γ and *RAR* β/γ null mutants die in utero or at birth from severe developmental defects that include a spectrum of severe malformations in the cardiovascular, central nervous systems and trunk (Wilson et al., 1990). *RXR*α knockout mice displayed cardiac malformations and die from cardiac

Figure 1.8. Schematic representation of RA nuclear receptor. A typical nuclear receptor is composed of several functional domains. The variable NH_2-terminal region (A/B) contains the ligand-independent AF-1 transactivation domain. The conserved DNA-binding domain, or region C, is responsible for the recognition of specific DNA sequences. A variable linker region D connects the DBD to the conserved E/F region that contains the ligand-binding domain as well as the dimerization domain. The ligand-independent transcriptional activation domain is contained within the A/B region, and the ligand-dependent AF-2 core transactivation domain within the COOH-terminal portion of the LBD. DBD, DNA-binding domain; LBD, ligand-binding domain; AF-1,2, activation function (adapted from Chambon 1996).


LBD
N
A/B
C
D
E
F
C
AF-1
AF-2
DBD

failure at about day 15 of gestation (Kastner *et al.* 1994; Sucov *et al.* 1994). The ventricular myocytes exhibit a premature differentiation, which can be visualized as early as 8.5 dpc, as well as a reduced rate of proliferation. Many cellular events are affected in the mutants including disruption of sarcomere assembly, cell cycle control, and control of cell-cell adhesion. *RXR* β null mutant mice are developmentally normal, but the adult males are sterile. Any compound mutants of RAR and RXR have more serious defects on embryonic development with the heart being one of the major organs affected (Zile 2004).

In addition to the nuclear receptors, there are additional RA-binding proteins. Two isoforms of a cytoplasmic cellular retinoic acid binding protein (CRABP I and CRABP II) have been described and their functions may include the protection of RA from oxidation, ligand solubilization, regulation of RA metabolism, regulation of RA access to cellular compartments such as the nucleus and coactivator function.

1.3.5.3 Mechanism of RA action

RA functions by binding to nuclear receptor proteins that activate or repress the transcription of downstream target genes. RARs and RXRs form a dimer that interacts with specific DNA sequences entitled the retinoic acid response elements (RARE) in the promoter region of target genes. This dimer binds the RARE in a complex regardless of whether RA is present or not. However, RAR-mediated activation of target genes involves the ligand-dependent dissociation of a corepressor complex and the association with a growing list of coactivator proteins (McKenna and O'Malley, 2002).

In one model of RA action, liganded RAR/RXR heterodimers first recruit coactivators and HAT complexes resulting in chromatin relaxation, which expedites entry

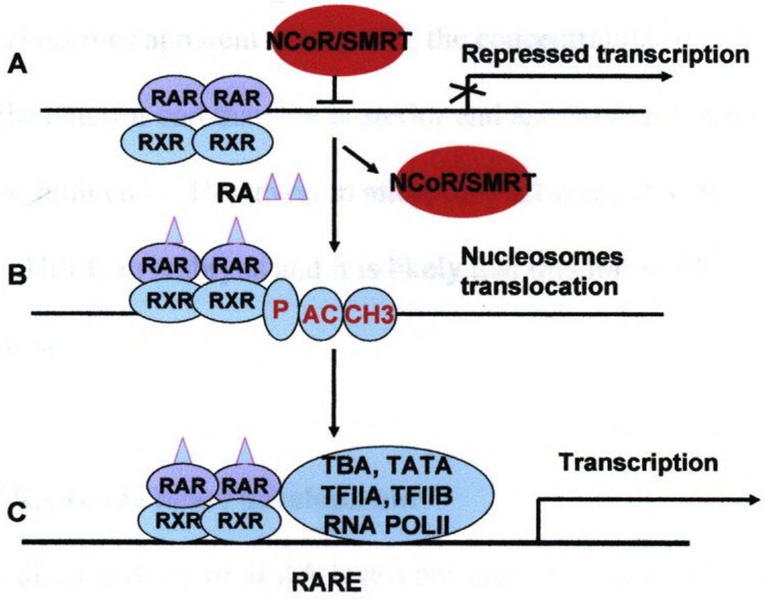
of the transcriptional machinery to the promoter through its interaction with the RNA Pol II holoenzyme (Woychik and Hampsey, 2002 and Dilworth and Chambon, 2001). Finally, the recruitment of general transcription factors is also enhanced by cAMP response element-binding binding protein (CBP) associated to the RNA Pol II holoenzyme (Adelman and Lis, 2002; Orphanides and Reinberg, 2002). Once transcription is initiated, RNA Pol II traffics along the gene to be transcribed. This process involves chromatin remodeling and modifying activities endowed by subunits of the elongation factors that track with elongating RNA Pol II (Orphanides and Reinberg, 2000). Finally, the equilibrium shifts in favour of deacetylation and methylation at residues leading to the rapid conversion of chromatin to a repressed conformation (Fig. 1.9).

1.3.5.4 The roles of RA in embryonic development

A variety of experimental approaches have been used to study the importance of RA signaling in developing embryos, including embryonic exposure to insufficient and excessive RA at various stages of development by maternal dietary manipulation and use of retinoid antagonists, as well as genetic approaches. The first evidence that demonstrated a role for RA in development came from the work of Hale in which the deficiency of RA could produce congenital malformations in the offspring of pigs. These defects include eyes, cleft palate, hare lip, accessory external ears, and the arrested ascension of the kidney. All defects were prevented by supplementing the control diet with RA (Zile 2002).

Mice that lacks RALDH2 die at E10.5 and display severe embryonic abnormalities including a lack of heart looping and chamber morphogenesis.

Figure 1.9. The three-step mechanism of retinoid receptor action. A, In the absence of RA, corepressors such as NcoR/SMRT bind to RA receptor complex and recruit HDAC repressing transcription. **B,** In the presence of RA, RA binds with RAR and induces conformational changes in the receptor. This causes a dissociation of corepressors and the coordinated recruitment of coactivators. The identified coactivators include SRC-1/NCoA-1, TIF-2/GRIP-1/NCoA-2/SRC-2, p300/CBP and CARM-1. They may phosphorylate, acetylate and methylate target genes resulting in chromatin relaxation. **C,** Once repressive chromatin has been decondensed, RNA-Pol II and general transcription factors (GTF) enter the preinitiation complex. Once transcription has been initiated, RNA Pol II traffics along the gene to be transcribed.
Ch3, methylation; P, phosphorylation; AC, acytelation, RNA Pol II, RNA polymerase I; NCoR, nuclear receptor repressor and SMRT, retinoic and thyroid hormone receptors; TFII, transcription preinitiation complex; TBA, termination site binding activity (adapted from Chen 2004).


A
NCoR/SMRT
Repressed transcription
RAR
RAR
RXR
RXR
RA
NCoR/SMRT
B
Nucleosomes
translocation
RAR
RAR
RXR
RXR
P
AC
CH3
C
RAR
RAR
RXR
RXR
TBA, TATA
TFIIA,TFIIB
RNA POLII
Transcription
RARE

region, and absence of limb buds. In *Xenopus* HPLC studies have revealed the presence of various retinoids, including all-*trans*-RA, 4-*oxo*-RA and 9-*cis*-RA, from fertilisation onwards (Durston et al., 1989). Spatial distribution studies have shown that, when the anteroposterior axis becomes apparent at stage 13, the concentration of all-*trans*-RA is two-fold higher at the anterior end than the posterior end and 9-*cis*-RA is absent in the middle but present at both ends. However, in mice, only all-*trans*-RA was found at significant levels by HPLC in embryos and it is likely that this form of RA is the only biologically relevant one.

1.3.5.5 The role of RA in the heart development

As has been discussed above, if RA levels are disrupted, there are severe consequences to cardiovascular development because RA affects all of the early signaling molecules regulating cardiac progenitor differentiation, including Wnts, FGF and BMPs. Wnt-5a, which inhibits heart development, is up-regulated by RA. RA also positively regulates FGF expression in the heart as deficiency in RA leads to a decrease in FGF expression (Zile 2004).

RA also significantly regulates the expression of the key cardiac transcription factors such as *Nkx2.5, GATA4* and *eHand*. For example, RA up-regulates *GATA4* expression in the posterior heart tube. In the VAD quail embryo *GATA4* expression is severely diminished in the cardiac inflow tract–forming area. However, addition of RA causes an upregulation of *GATA4* in *Xenopus* and rescues cardiac formation.

In addition to affecting cardiac morphogenesis, RA also limits the pool of cardiac progenitors in the cardiac forming region. Loss of function experiments in mammalian

embryos created by genetic ablation of RA receptors or RALDH2 (Niederreither et al., 2001) have shown that RA is necessary for normal cardiacmorphogenesis and anterior–posterior patterning of the heart tube (Iulianella and Lohnes, 2002).

The embryonic heart tube is patterned prior to looping (Pandur et al., 2002; Schneider and Merola, 2001). Administration of excess RA to chick embryos truncates the anterior portion of the heart tube and enlarges its caudal portion (Niederreither 1998). The posterior most segments of the heart tube (putative atria and sinus venosus) are markedly reduced in size in *RALDH2-/-* embryos. Furthermore, expression of *Tbx5*, which marks the prospective atria and sinus venosus, is reduced and shows an ill-defined posterior boundary in the mutants. In addition, excess RA can result in either lack of fusion of the paired cardiac primordia, and/or truncation of the atrium with abnormal expansion of ventricular structures (Drysdale et al., 1997).

1.4 Purpose of the Thesis

Retinoic acid is an important signaling molecule known to be essential in patterning and morphogenesis of the heart formation, but excess RA can inhibit *Nkx2.5* gene expression and block myocardial differentiation, as assayed by *cTnI* expression (Drysdale et al., 1997).

In order to investigate the mechanism by which excess RA inhibits *Nkx2.5* expression and blocks myocardial differentiation, we compared gene expression patterns induced by the addition of RA and RA antagonist in *Xenopus* using Affymetrix© GeneChip technology. Our results show that RA increases *FS* expression and decreases *Tbx20* expression. FS is an antagonist of both activin and BMP and BMP signaling is

required for cardiomyocyte differentiation. Tbx20 is an important cardiac transcription factor and loss of Tbx20 function causes heart tube abnormalities.

Based on our Affymetrix© GeneChip results, we developed our hypothesis that increased *FS* gene expression by RA may be responsible for the inhibition of the heart formation through opposing BMP activity.

Our whole mount *in situ* hybridization results have confirmed the up-regulation of FS gene expression by RA and down-regulation by RA antagonist in the heart-forming region. Increased follistatin can inhibit *Nkx2.5* expression. My rescue experiments showed that BMP and activin can reverse the inhibitory effects of the heart formation by FS. Thus, we first presented evidence to demonstrate that an increase in *FS* gene expression by RA is responsible for the inhibition of cardiomyocyte differentiation by decreasing BMP signaling, which is required for the heart formation.

Our whole mount *in situ* hybridization also confirmed that RA inhibits *Tbx20* expression in the developing heart. BMP can rescue the inhibitory effects of RA on *Tbx20* expression which indicates that RA also inhibits *Tbx20* expression by decreasing BMP signaling.

We investigated the effects of RA on *BMP2, 4* and *7* expression so that we can understand the mechanisms by which excess RA decreases BMP signaling. Our results showed that RA does not significantly affect BMP expression. This suggests that RA does not alter BMP signaling at the level of transcription of the ligand.

We conclude that excess RA inhibits cardiomyocyte differentiation at least partially through increasing follistatin expression.

CHAPTER 2 MATERIALS AND METHODS

2.1 *Xenopus laevis* – Drysdale Colony

Adult *Xenopus laevis* male and female frogs were purchased from *Xenopus* I, Michigan, USA and housed at the Victoria Research Laboratories animal facility. All animals were treated according to protocols approved by the Animal Care Committee at the University of Western Ontario according to the regulations stipulated by the Canadian Council on Animal Care.

2.2 Embryo production and culture

Embryos were generated as in Drysdale and Elison (1991), with minor changes to be reported. *Xenopus. laevis* males were sacrificed, testes were isolated and stored in 200% Steinberg's solution (2000% stock; 1.16 M NaCl, 13.4 mM KCl, 6.8 mM $Ca(NO_3)_2.4\ H_2O$, 16.6 mM MgSO4.$7H_2O$, 92.5 mM Tris-HCl (pH 7.4)) at 4 °C. *Xenopus laevis* females were injected with 700-800 IU of hCG (Sigma, St. Louis, MO, USA) and incubated for 16 hr at 17 °C. Ooctyes were manually squeezed into Petri dishes (VWR, West Chester, PA, USA) and fertilized *in vitro* using testes minced in 80% Steinberg's solution. To maximize fertilization rates, oocytes were immediately manipulated into a monolayer and allowed to remain undisturbed for 20 min. To hydrate jelly encompassing the eggs and facilitate their separation process, Petri dishes were next flooded with 20% Steinberg's solution. Embryos were dejellied at 2-4 cell stage with 2.5% cysteine, pH 8.0. To ensure embryo viability, excess cysteine was flushed away by repeated rinsing with 20% Steinberg's solution, where they developed at 17-22 °C until the appropriate

stage for treatment. All embryos were staged according to Nieuwkoop and Faber's Normal Table of *Xenopus laevis* (Nieuwkoop and Faber 1967).

2.3 Experimental Compounds

Retinoic acid (all-trans; Sigma) was prepared as 1 mM stock solution in DMSO and stored at -70 °C. Embryos were cultured in 20% Steinberg's containing RA at a concentration of 1μM. Our RAR-α specific RA antagonist, AGN194301, was prepared as a 1 mM stock solution in DMSO and stored at -70 °C. It was provided to our lab via a Materials Transfer Agreement with Vitae Pharmaceuticals Inc (Fort Washington, PA, USA). Embryos were cultured in 20% Steinberg's containing AGN 194301 at a concentration of 1μM.

2.4 Experimental Treatments

2.4. 1 Treatment

At stage 14, embryos were treated with 1 μM RA, 1 μM RA antagonist (AGN 194301) and 1 μM DMSO as control. At stage 20, some embryos were fixed for whole mount *in situ* hybridization and the others continued to develop until stage 32. They were also fixed for whole mount *in situ* hybridization.

2.4. 2 GeneChip sample preparation and analysis of data

Total RNA samples from *Xenopus laevis* at st 20 was isolated and submitted to the London Regional Genomics Centre for GeneChip analisis. Images of the hybridized arrays were imported into GeneSpring 7.0 software for statistical analysis.

We set stringent criteria for screening these genes. First, the fold change must be larger than 1.5. Second, the changes in gene expression must happen twice in our triplicate experiments. And last, the gene expression change between RA and RA antagonist must be opposite. All the genes whose expression pattern meets the following criteria are included in the table.

2.4. 3 Explant experiment

Also at stage 14, explants were isolated from anterior-lateral plate mesoderm where the heart-forming regions have been identified. The explants were cultured in 1X MBS solution (88 mM NaCl, 1 mM KCl, 0.7 mM CaCl, 1mM $MgSO_4$, 5 mM HEPES (pH 7.8) and 2.5 mM $NaHCO_3$) until analyzed at stage 20 by whole-mount *in situ* hybridization.

Follistatin (National Hormone and Pituitary Program, Bethesda, MD) was prepared as a 1 mg/ml stock in H_2O and diluted to working concentrations. Human recombinant activin A (National Hormone and Pituitary Program), and human recombinant BMP4 (Genetics Institute, Cambridge, MA) were diluted to working concentrations in H_2O. Solutions of BMP, activin and follistatin were prepared immediately prior to use. Follistatin, BMP and activin concentrations were consistent with previously published work (Lough *et al.,* 1996).

FS, BMP4 and activin were added to the cultured explants medium at a concentration of 20 ng/ml and the treatments were continuous until the embryos were fixed for *in situ* hybridization to examine *Nkx2.5* and *Tbx20* mRNA. Some explants were treated with ketoconazole to examine the effect of endogenously increased RA on *Nkx2.5*

gene expression. Ketoconazole was prepared as a 1 mg/ml stock in H_2O and diluted to working concentrations of 10 ng/ml.

Different stage embryos were placed in MEMPFA [4% paraformaldehyde, 1 mM $MgSO_4$, 2 mM EGTA pH 8.0), 0.1 M MOPS (pH 7.4)] for either 2 hr at RT or O/N at 4 °C. Embryos were then dehydrated through a graded methanol series (25%, 50%, 75%, 100%) and stored at -20 °C.

2.5 Plasmid Transformation

100 µl of chilled competent cell culture was mixed with 1 ng of plasmid to be transformed, described below. These mixtures were then heat-shocked at 37 °C for 90 sec and subsequently placed on ice for a minimum of 15 min. Positive and negative controls were always employed. Solid LB (5 g bacto-tryptone, 2.5 g bacto-yeast extract, 5 g NaCl & 7.5 g bacto-agar, per 500 ml) plates containing ampicillin (1 ng/ml) were used to select for potential transformats. In brief, approximately 80 µl of an individual transformation reaction was aliquoted atop a solidified LB-ampicillin plate and spread out evenly with the assistance of an alcohol sterilized glass wand. Plates were incubated face down overnight at 37 °C and analyzed for transformats in the morning.

2.6 DNA Plasmid Preparation

Plasmid DNA was prepared using Qiaprep miniprep kits as the manufacturer's description. To elute DNA, add 50 ul buffer or water to the center of each QIAprep spin column, let stand for 1 min and centrifuge for 1 min. Eluted DNA was stored at -20 C for future use.

2.7 Digoxygenin-labeled antisense RNA probe generation

Antisense digoxygenin-labelled RNA probes were transcribed in vitro using cDNA clones *XFS* (GB S69801), *XTbx20* (AY154394) from ATCC, *XNkx2.5* (Tonissen et al., 1994), *XBMP2* (Suzuki et al., 1997), *XBMP4* (Nishimatsu et al., 1992), *XBMP7* (Suzuki et al., 1997) and *XTnIc* (Drysdale et al., 2003).

Linearized templates were produced by digesting 10 µg of each plasmid with its appropriate restriction enzyme and buffer for 2 hr at 37 °C. The transcription reaction was assembled by adding 1-2 µg of template DNA to 5.5 µl dH_2O, 4 ul digoxygenin labelled NTP mixture (10 mM ATP, 10 mM GTP, 10 mM CTP, 6.5 mM UTP (Pharmacia), 3.5 mM digoxygenin coupled 11-UTP (Roche)), 4 µl 0.1 M DTT, 4 ul 5X transcription Buffer (MBI Fermentis: 200 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 10 mM spermidine-HCl), 0.5 µl RNAguard inhibitor (Pharmacia) and 2 ul of RNA polymerase (MBI Fermentas; 15-20 units/µl) and incubated at 37 °C for 2 hr. Removal of template DNA was achieved by adding 1 µl of DNAse I (Life Technologies) to the reaction and performing an additional 37 °C incubation for 10 min.

1 µl of the transcribed RNA was next analyzed through gel electrophoresis. RNA was run in a 1% TAE-agarose gel containing ethidium bromide and visualized by UV illumination. RNA pellets were resuspended into 500 µl of RNA Hybridization Buffer (50% formamide, 5X SSC (20X stock; 3 M NaCl, 300 mM $NaCHO_3$, pH 7.0), 5 mM EDTA (pH 5.0), 1 mg/ml purified Yeast RNA (Roche), 1 M Denhart's solution (2% BSA, 2% PVP-40, 2% Ficoll 40), 0.1% Tween20 and 100µg/ml heparin (Sigma). Probes were further diluted with an additional 5-10 ml of RNA Hybridization Buffer and stored indefinitely at -20 °C.

2.8 Whole-mount *in situ* Hybridization

Whole-mount *in situ* hybridization was performed as previously described by Harland (1991), with several modifications. Vials were placed onto a nutator in order to provide adequate movement for liquid infiltration. Approximately 10 embryos from each treatment group were aliquoted into separate vials containing 2 ml 100% methanol. Rehydration of *Xenopus* embryos, achieved using a graded methanol series (100%, 75%, 50%, 25%) and was followed by two, 2 min rinses with 2 ml of TTW (50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 0.1% tween-20). In order to assist with riboprobe penetration, embryos were treated for 5 min in 2 ml of TTW containing 20 mg/ml proteinase K (Sigma). Embryos were refixed in MEMPFA for 20 min followed by three 5 min washes in 4 ml TTW. Embryos were prehybridization for 10 min at RT and then one hr at 65 °C. Next, 2 ml of the appropriate digoxygenin-labeled riboprobe was added and the embryos were incubated overnight at 65 °C.

The following morning, embryos were subjected to a series of rinses to remove excess probe, reduce background levels and prepare for antibody addition. After the riboprobes were recollected and placed back at 20 °C, a 10 min incubation with 4 ml RNA hybridization Buffer at 65 °C was performed, followed by two, 20 min rinses 2X SSC at 37 °C. Unbound riboprobe and single-stranded RNA was then degraded by an additional 30 min wash using 2X SSC containing 5 µg/ml RNAse (ribonuclease; MBI Fermentas) at 37 °C. Next, three,1 hr washed with 0.2X SSC/0.1% Tween-20 at 65 °C served to eliminate trace amounts of riboprobe trapped non-specifically within embryonic cavities. Both the embryos and a 1:4000 dilution of alkaline phosphatase-conjugated anti-digoxygenin Fab fragments (Roche; 0.75 U/ul) were blocked for 1 hr at 4 °C in a 0.1 M

MAB (100 mM maleic acid, 150 mM NaCl) containing 4% HTLS (Heat-treated lamb serum) and 2% blocking reagent (Bohringer Mannheim). Then blocked antibody solution was subsequently added to the blocked embryos and incubated overnight at 4 °C.

To remove all excess antibody, the third day involved at least ten, 30 min MAB washes. To maximize the removal process, 6 ml of MAB was added to each tube and the embryos were allowed to shake horizontally. These washes were followed by 5 min incubation in alkaline phosphatase buffer (100 mM NaCl, 100 mM Tris-HCl (pH 9.5), 50 mM $MgCl_2$, 0.1% Tween20). Alkaline phosphatase staining was initiated upon the addition of 0.5 ml alkaline phosphatase substrate BM purple (Roche, Germany) to each vial. Embryos were then incubated at RT until visual observation of alkaline phosphatase activity was evident. The length of this step depends on a multitude of factors including the probe quality, mRNA transcripts abundance, fixation period and overall handling of the embryos.

Staining can be detected within 4-5 hrs but may take upwards of 72 hrs to appear. Typically, phosphatase staining was detected approximately 24 hrs after the addition of BM purple. The colour reaction was stopped via dehydration and rehydration of the embryos using a graded methanol series (25%, 50%, 75%, 100%). Embryos were counterstained yellow by overnight incubation in 2 ml Bouin's Fixative (per 100 ml, 1 g picric acid, 25 ml glacial acetic acid). Embryos were fixed for 15 minutes in MEMPFA and again dehydrated through a graded methanol series for storage at -20 °C.

2. 9 Statistics

All values are presented as means ± SEM. Differences between treated and

control groups were analyzed using ANOVA paired test. A value of $P < 0.05$ was considered statistically significant.

CHAPTER 3 RESULTS

3. 1 The effect of RA and RAA on gene expression

To understand the mechanism of RA inhibition of *Nkx2.5* expression and myocardial differentiation, I used Affymetrix GeneChip® technology to examine changes in gene expression caused by the addition of RA or an RA antagonist in the early *Xenopus* embryos. The *Xenopus leavis* Genechip® contains 14, 400 probe sets (http://www.affymetrix.com/index.affx), enabling an unbiased examination of a large number of potential gene changes.

In *Xenopus*, cardiomyocyte specification takes place shortly after gastrulation (Shi et al., 2000; Sater and Jacobson 1989). Previous experiments have shown that excess RA inhibits cardiomyocyte differentiation when embryos were treated with 1 μM RA shortly after gastrulation (Drysdale et al., 1997). Alternatively, if RA signaling is blocked for four hours at this stage, differentiation appears normal but it will result in a significant inhibition of the ability of myocardial progenitors to form a tube (Collop et al., 2006). It should be noted that addition of RA for the same four hour window causes a significant decrease in myocardial differentiation (Collop et al., 2006).

Therefore, we selected the same treatment point (stage 14) and RNA extraction at stage 20 (four hours later) for our analysis of gene expression. Analysis using GeneSpring 7.1 software demonstrated that there were 20 genes whose expression was up-regulated or down-regulated by at least 1.5 fold under treatments with RA or the RA antagonist. After stringent screening, we got 10 genes and put them on the table1. Table 1 lists those genes whose expression met the above criteria.

As discussed previously, CYP26 and RALDH2 are two key enzymes in controlling the levels of RA since the former is responsible for RA degradation and the latter is responsible for RA synthesis. It is known that excess RA induces *CYP26* expression and decreases *RALDH2* expression while RA antagonist treatments decrease *CYP26* expression and increase *RALDH2* expression (Duester et al., 2003). Expression profiles of these two key enzymes in our GeneChip® results were consistent with previous studies, suggesting that our dataset is valid.

RA signaling up-regulates *follistatin* expression in p19 cells (Fainsod et al., 1997), but, to date, there are no reports that RA can increase *follistatin* expression in whole embryos. Because of follistatin potential for inhibiting BMP, an important mediator of heart development, we decided to investigate it further. We used whole mount *in situ* hybridization to validate the putative expression changes predicted by the microarray. Whole mount *in situ* hybridization was used for verification because we predicted that changes in the size of the expression domain would be the primary cause of the changes in expression levels. Our results have confirmed the changes in *follistatin* (Figure 3.1) and *Tbx20* (Figure 3.2) expression due to altered levels of retinoic acid signaling.

3.2 The developmental changes in FS gene expression

When we observed expression in *follistatin*, a marked change was noted on the ventral side at stage 20. In control embryos, there was virtually no *follistatin* expression on the ventral side, but when embryos were exposed to RA, *follistatin* expression was very obvious (Fig. 3.1). As previous studies did not describe *follistatin* expression on ventral side (Hemmati-Brianlou et al., 1994), we decided to re-examine

Table 1. Genes induced in RA and RAA treated embryos from GeneChip analysis

The RA-regulated genes are included in this table. We set stringent criteria for screening these genes. First, the fold change must be larger than 1.5. Second, the changes in gene expression must happen twice in our triplicate experiments. And last, the gene expression change between RA and RA antagonist must be opposite. All the genes whose expression pattern meets the following criteria are included in the table.

The values in fold change represent the average of two or three experimental results. Symbol "+" indicates fold increase and "-" indicates fold decrease. The symbol "a" indicates prior published evidence for RA-stimulated gene. The symbol "b" indicated that this was not previously reported, but validated by our whole mount *in situ* analysis.

GenBank No	Name	Average fold change by RA	Average fold change by RAA	Function	Validation
BJ088393	Cyp26	+14.6	-5.3	Metabolizing RA	a (Ross, 2003)
CB560503	Short-chain dehydrogenase reductase	+ 9	-3.5	Interconversion of retinol to retinal	a (Duester 1996).
AB014463	Xenopus est	+5	-2.3	unknown	Not validated
BU905390	Transcription factor HNF-1^	+4.3	-2.9	Cell differentiation	a (Kuo et al., 1991)
BJ088899	HoxA1	+4.3	-7	Hindbrain segmentation and specification	a (Wendling et al., 2000)
S69801	Follistatin	+2.9	-2.7	Antagonist of BMP and activin	b
BJ091549	Phosphorylase kinase	+1.9	-5.4	identified	Not validated
AF310252	RALDH2	-6	+3	RA synthesis	a (Maden 2006)
AY154394	Tbx20	-2.3	+1.6	Transcription factor	b
AF283102	Nkx2.5	-1.9	+1.7	Cardiac transcription factor	a (Drysdale et al., 1997)

Figure 3.1. The effects of RA and RAA on *FS* gene expression. Different levels of RA significantly affect *FS* expression. At embryonic stage 20, *FS* is normally expressed on the dorsal side (B) and weakly on the ventral side (E). When embryos were treated with RA, *FS* expression domain is enlarged on both the dorsal side (C) and ventral side (F). For the embryos treated with RA antagonist, there is no detected FS staining on the ventral side (D) and less staining on the dorsal side (A) compared with control embryos. The entire experiment was performed in triplicate, with at least 10 embryos per treatment being analyzed for *in situ* hybridization each time. Arrows point at the kidney (upper panel) and the heart (lower panel).

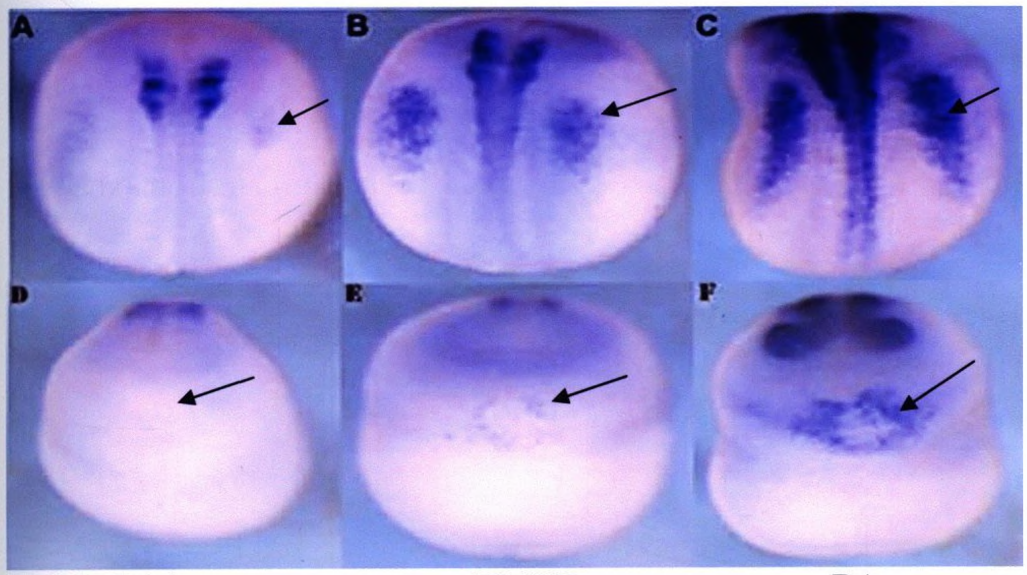
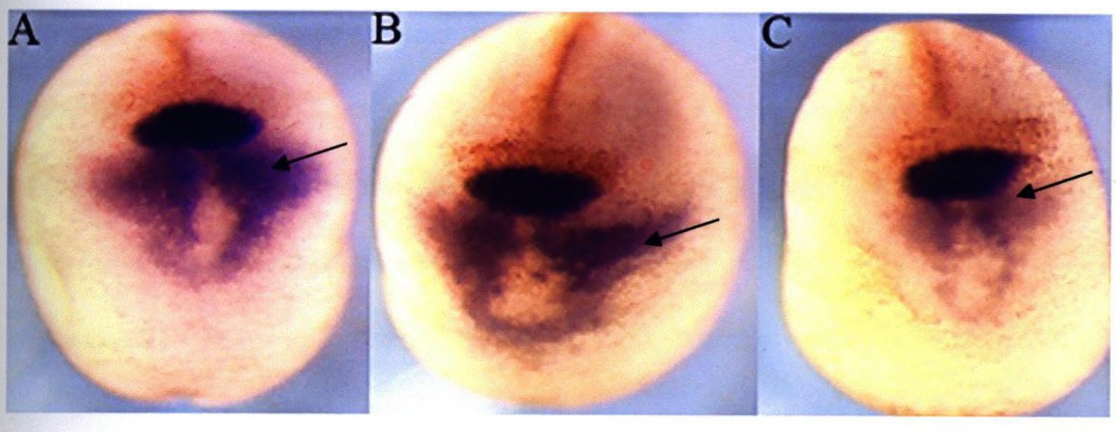

A
B
C
D
E
F
Dorsal view
Ventral view
RAA
DMSO
RA

Figure 3.2. The effects of RA on *Tbx20* gene expression on whole embryos

Excess RA significantly affects *Tbx20* expression. Treatments of embryos with RA (1 μM) and RA antagonist (1 μM) at stage 14 alter the *Tbx20* expression domain. At embryonic stage 20, *Tbx20* is normally expressed in a subdomain of the cement gland and the developing myocardium immediately posterior to the cement gland (B). When embryos were treated with RA at stage 14, the expression of *Tbx20* domain is diminished (C). There is little difference in the *Tbx20* size of expression domain between control (B) and RA antagonist treated embryos (A). The entire experiment was performed in triplicate, with at least 10 embryos per treatment being analyzed for *in situ* hybridization each time. Embryos are oriented with ventral views and anterior to the top. Arrows point at the heart.



RAA **DMSO** **RA**

its expression on the ventral side at multiple stages of development.

Using *in situ* hybridization, *follistatin* expression was first found in the organizer of the *Xenopus* embryo at stage 12, as previously observed (Smith and Harland, 1992; Hemmati-Brivanlou et al., 1994; Sasai et al., 1995). From stage 12 to stage 32, *follistatin* expression is observed in anterior notochord, midbrain, hindbrain, eye, pronephros, and blood islands (Fig. 3.3). Previous reports describe the same temporal and spatial patterns of *follistain* expression (Hemmati-Brivanlou et al., 1994), however, early reports did not describe *follistatin* expression near the developing heart. We observed *follistatin* expression posterior to the cement gland on the ventral side at stage 20 (Fig. 3.4). As development proceeds, *follistatin* expression is observed on the ventral side near the presumptive heart-forming region (Fig. 3.4). *Nkx2.5* and *Tbx20* are early markers of presumptive cardiomyocytes and *cTnI* is marker of differentiated myocardium. When comparing these three gene expression patterns, we can clearly see that *follistatin*, *Nkx2.5*, *Tbx20* and *cTnI* are expressed in close proximity *in vivo* (Fig. 3. 4).

3.3 The effects of RA on FS gene expression in the heart-forming region

When the areas of *follistatin* expression domain are compared at stage 20, we found that the *follistatin* expression domain in RA treated embryos is larger than that in control embryos (Fig. 3.1). This is also obvious in other expression domains of follistatin. In addition, when the distance between the edge of *follistatin* expression domain and cement gland is compared, the control embryos have a noticeable gap between the *follistain* expression domain and the cement gland (Fig. 3.1). In RA-treated embryos, the *follistatin* expression domain is adjacent to the cement gland. In RA antagonist

Figure 3.3. The developmental changes in FS gene expression. *FS* gene expression patterns are examined by whole mount *in situ* hybridization. At stage 12 (A) *FS* expression begins to appear in prechordal plate and anterior notochord. At stages 14 (B), 18(C), 20 (D), 22 (E), 28 (J) to 32 (K), *FS* expression is obvious in anterior notochord, midbrain, hindbrain, eye and pronephros. From stage 18 (G), *FS* begins to be expressed in a few cells of the ventral mesoderm. At stages 20 (H) and 22 (I), *FS* expression appears in the heart-forming region. At stage 28 (J) and 32 (K), *FS* is still expressed in the heart. Embryos are oriented with dorsal side up to the top (A, B, C, D, E), the anterior to the top (F, G, H, I), the anterior to the left (J, K). an, anterior notochord; mb, middlebrain; n, notochord; p, pronephros; b, blood island and h, heart. G is ventral view.

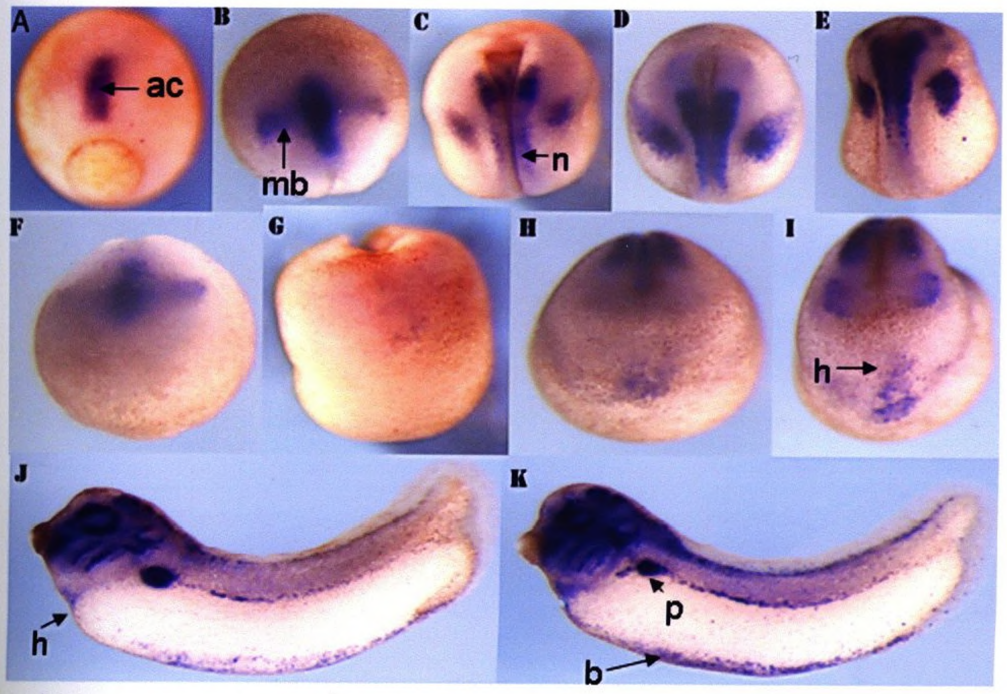
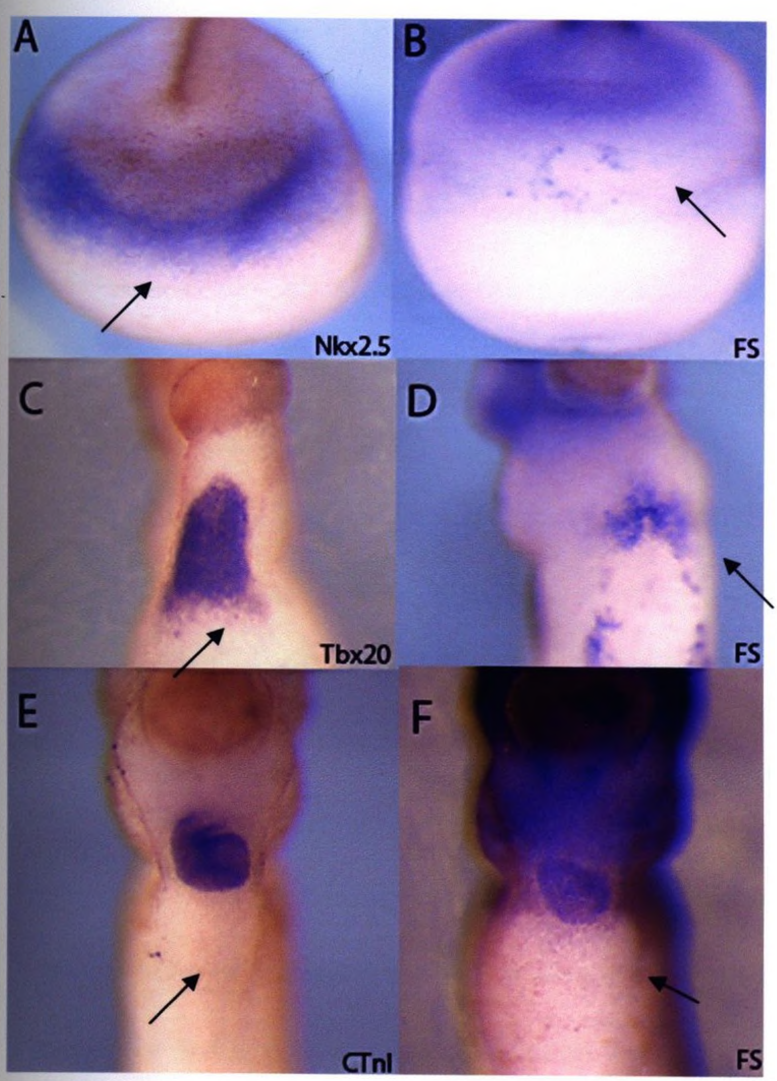

A
ac
B
mb
C
n
D
E
F
G
H
I
h
J
h
K
p
b

Figure 3.4. FS gene expression in the heart-forming region during development

FS, Nkx2.5, Tbx20 and *cTnI* are expressed in adjacent expression domains *in vivo*. Whole mount *in situ* hybridization shows the expression domains of *follistatin* at stage 20 (B), stage 26 (D) and stage 32 (F) when compared to the expression domain of *Nkx2.5* at stage 20 (A), *Tbx20* at stage 26 (C) and *cTnI* at stage 32 (F). It is clear that while not identical, there are somewhat overlapping expression between the myocardial markers *Nkx2.5, Tbx20, cTnI* and *FS*. Embryos are oriented with ventral views and the anterior to the top. Arrows point at the heart.


A
Nkx2.5
B
FS
C
Tbx20
D
FS
E
CTnI
F
FS

treated embryos, there is almost no detectable *follistatin* expression on the ventral side at this stage (Fig. 3.1). These results demonstrate that RA increases *follistatin* expression on the ventral side, as has been previously observed in P19 cells (Fainsod et al., 1997) and any changes in level of RA can significantly alter *follistatin* expression. It can also be concluded that RA is necessary for maintaining normal *follistatin* expression since the RA antagonist reduces *follistatin* expression.

3.4 The effects of FS on cardiomyocyte differentiation

Our initial hypothesis that excess RA inhibits heart development through increasing *follistatin* expression predicts that addition of follistatin to the heart region will inhibit cardiac differentiation. Therefore, we examined the effect of follistatin on *Nkx2.5* gene expression in cultured explants. Follistatin cannot penetrate to the heart-forming region because it cannot pass through the overlying ectoderm. However, when explanted, the cardiac mesoderm is exposed to the medium allowing for direct exposure to the follistatin.

The heart-forming region explants were removed from stage 14 embryos and cultured in MBS containing follistatin, BMP4 or activin (20 ng/ml for each). This concentration of follistatin or BMP or activin has been extensively used in previous studies for investigations into regulation of the growth factors (Ladd et al., 1998; Lough *et al.,* 1996). Each group contained 10 explants and they were cultured until their sibling embryos developed to stage 20 at which time they were fixed. After fixation, whole mount *in situ* hybridization was performed and the average area of the *Nkx2.5* expression domain was measured. Our results showed that *Nkx2.5* expression domain is significantly

smaller in explants treated with follistatin than that in control explants. Importantly, addition of BMP restores the size of the *Nkx2.5* expression (Fig.3.5).

Since activin is a FS antagonist, we examined the effects of FS on *Nkx2.5* expression in the presence of activin. The same explant procedure as before was used except that the explants were cultured in the presence of activin (20 ng/ml) and FS (20 ng/ml). These results demonstrate that activin can rescue the inhibitory effects of FS on *Nkx2.5* expression (Fig. 3.5).

3.5 BMP and activin can rescue the inhibitory effect of RA on myocardial differentiation

We hypothesized that RA inhibits cardiomyocyte differentiation through inhibition of BMP signaling. If correct, this hypothesis predicts that addition of BMP should rescue the inhibitory effects of RA. As in the above experiment, explants were used to allow direct exposure of the BMP to the developing myocardium. The explants were cultured with 1 μM RA or RA plus BMP (20 ng/ml) or RA plus activin (20 ng/ml). Each group contained 10 explants. Four hours later, *Nkx2.5* expression was examined by whole mount *in situ* hybridization. As predicted (Drysdale et al., 1997), RA significantly decreases *Nkx2.5* expression domain (Fig.3.6). In RA treated explants, the average area of *Nkx2.5* expression domain is significantly reduced while addition of BMP rescued the inhibitory effects of RA (Fig.3.7). These results demonstrate that BMP can rescue the inhibitory effect of excess RA on *Nkx2.5* gene expression in cultured explants experiments.

Our previous experiments have demonstrated the possibility that RA inhibits

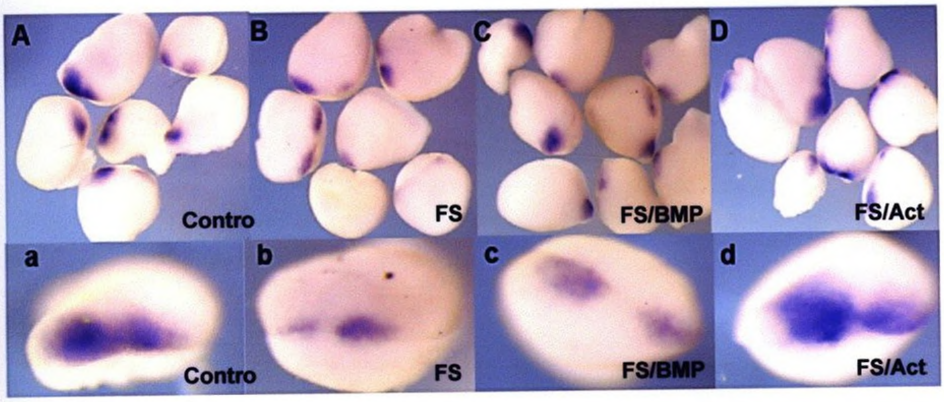
Figure 3.5. The effect of FS on the *Nkx2.5* expression domain in explants

I, When compared to control explants of the heart region (A and a), addition of FS reduces the size of the expression domain of *Nkx2.5* (B and b). In the presence of BMP (C and c) or activin (D and d), *Nkx2.5* expression domain increases compared to FS-only treated explants (B and b).

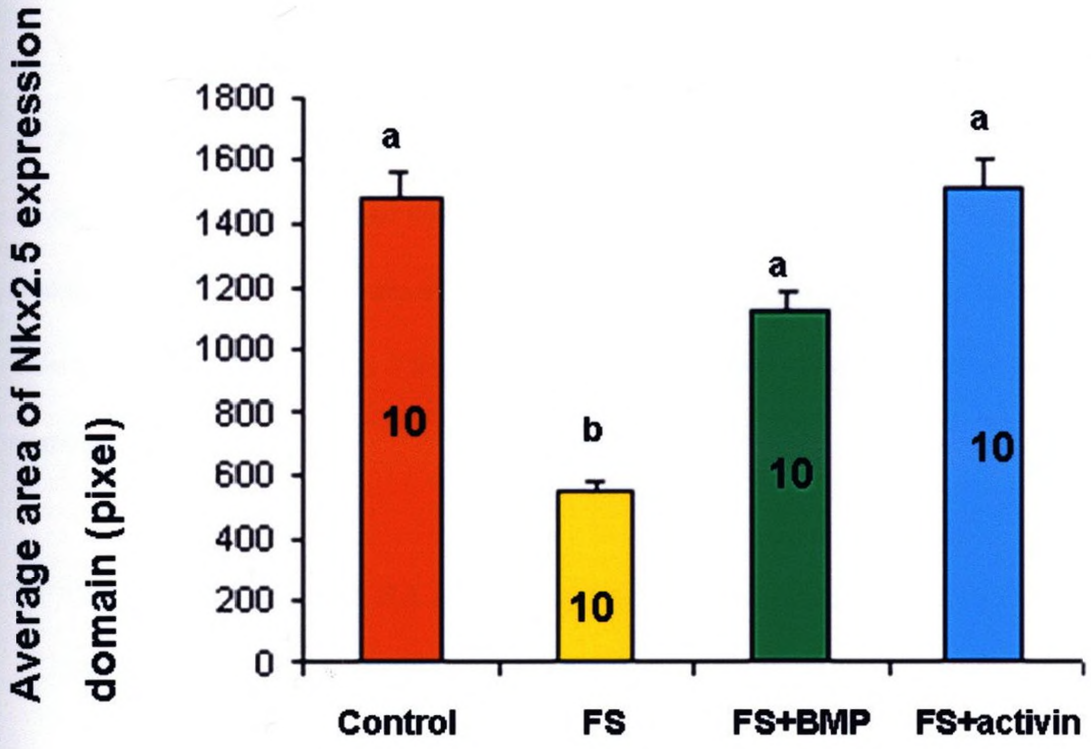
II, Quantified results are shown.

The same letters indicate no differences statistically. n value in box indicates explant number for measurement.

I



II


Average area of Nkx2.5 expression domain (pixel)
1800
1600
1400
1200
1000
800
600
400
200
0
a
b
a
a
10
10
10
10
Control
FS
FS+BMP
FS+activin

cardiomyocyte differentiation through increasing *follistatin* expression. Increased follistatin inhibits BMP signaling. In order to test the role of follistatin, we performed rescue experiments using activin, which is an antagonist of follistatin. If activin can rescue the inhibitory effects of RA on *Nkx2.5* expression, this will further demonstrate that follistatin is involved in RA-induced inhibition of cardiogenesis. Our results show that addition of activin rescued the inhibitory effects of RA (Fig.3.7).

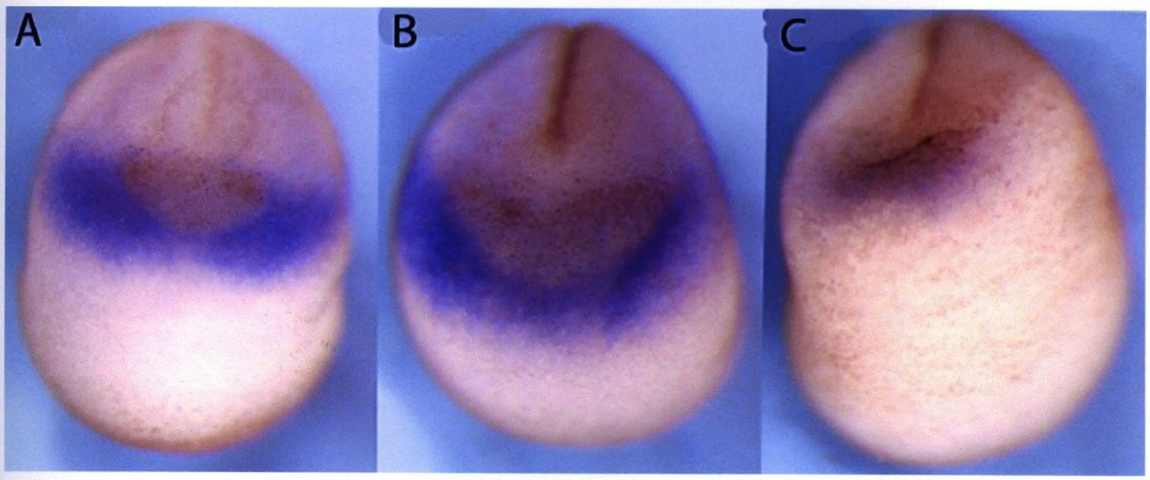
Our experimental results show that RA increases *follistatin* expression, therefore, we hypothesized that when low levels of FS and RA were added together, there would be an increased inhibitory effect by adding both FS and RA than used on their own. Our results showed that low levels of RA (0.1 μM) and FS display greater inhibitory effect than used individually (Fig. 3.8), indicating the inhibitory effect is additive when both of them were added together.

3.6 The effects of RA and RA antagonist on Tbx20 gene expression in the heart-forming region

Tbx 20 is an important cardiac transcription factor and is involved in early heart development (Takeuchi et al., 2005). Our microarray results suggested that *Tbx20* expression is also down-regulated by RA and up-regulated by RA antagonist. This was confirmed by our *in situ* hybridization results (Fig. 3.2). The fact that *Tbx20* and *Nkx2.5* are both rapidly down-regulated by retinoic acid suggests that they may share common regulatory mechanism.

To test this possibility, we wanted to determine if RA also inhibits *Tbx20* expression in a BMP dependent manner. Therefore, we carried out the same rescue

Figure 3.6. The effects of RA and RAA on the *Nkx2.5* expression. RA significantly decreases *Nkx2.5* expression in the heart-forming region. When compared to control embryos (B), RA reduces the size of the expression domain (C). When embryos were treated with RA antagonist, there is no significant change in *Nkx2.5* expression domain (A). In each case, the ventral pole of the embryo is shown, with anterior to the top.



RAA **DMSO** **RA**

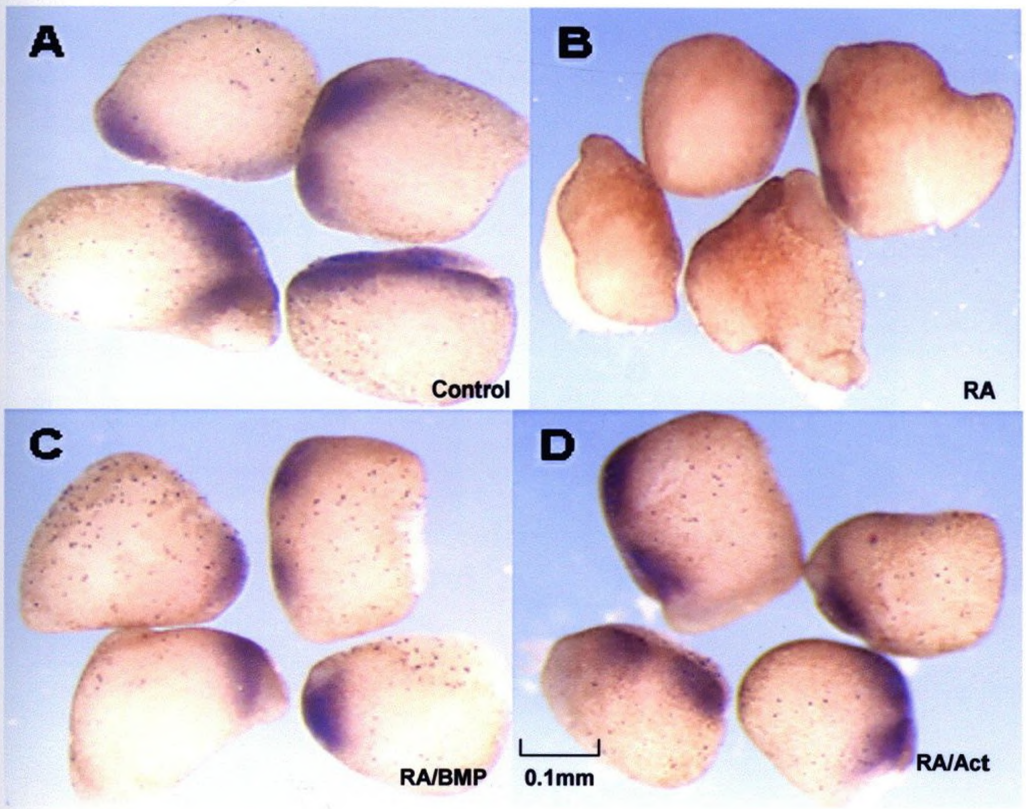
Figure 3.7. BMP and activin rescue the inhibitory effects of RA on Nkx2.5

I, When compared to control explants of the heart region (A), addition of RA reduces the size of the expression domain of the *Nkx2.5* (B). In the presence of BMP (C) or activin (D), *Nkx2.5* expression domain increases compared with only RA treated explants (B).

II, Quantified results are shown.

The same letters indicate no differences statistically. n value in box indicates explant number for measurement.

I



II

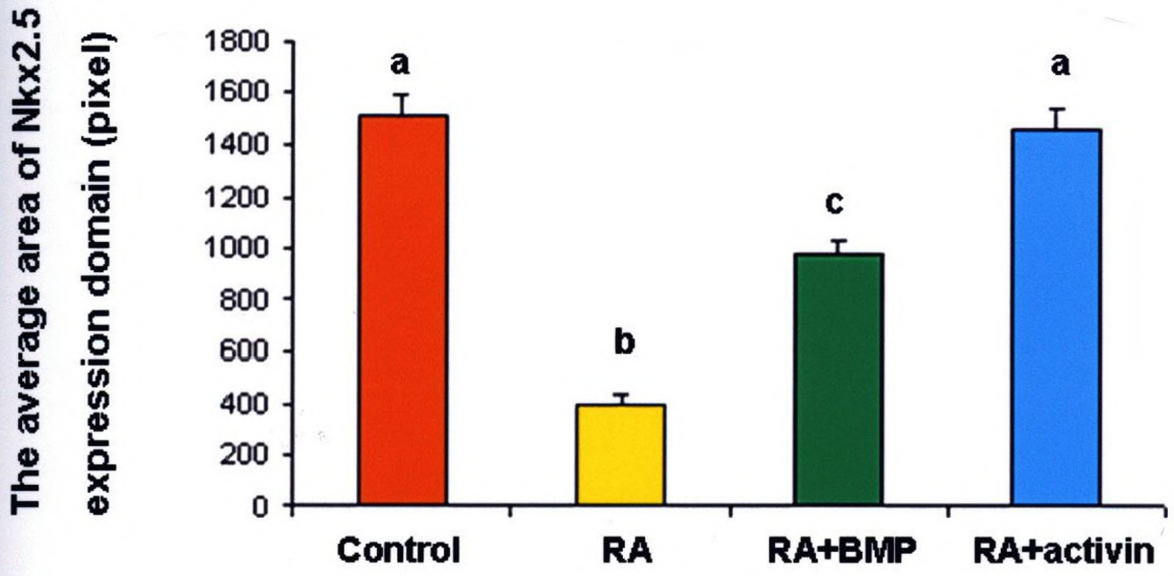
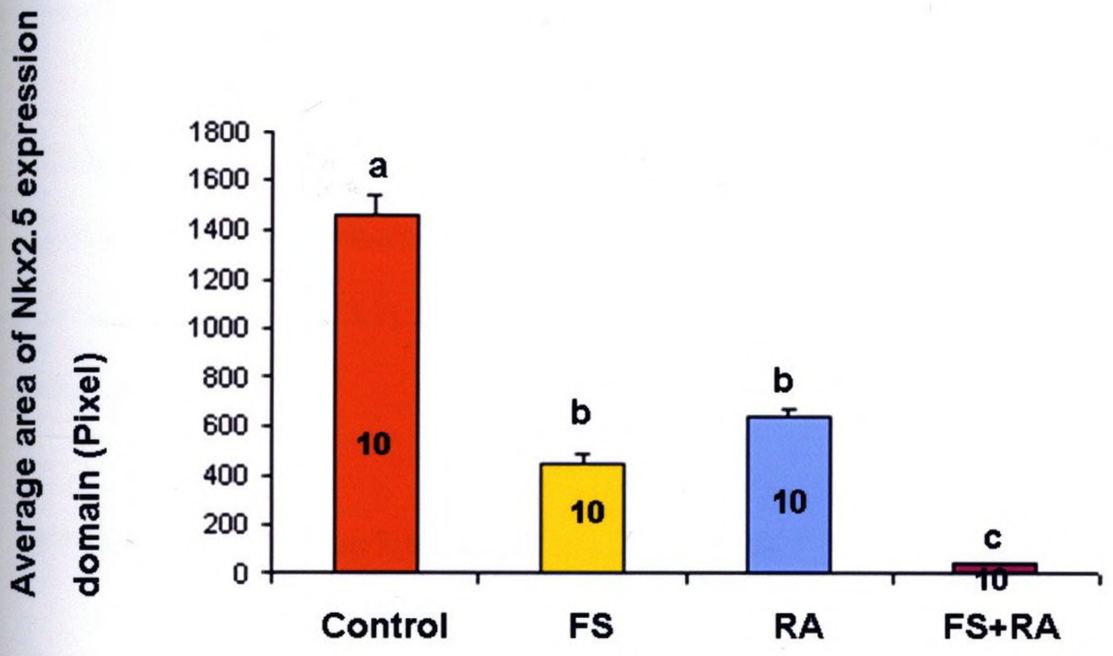

The average area of Nkx2.5 expression domain (pixel)
1800
1600
1400
1200
1000
800
600
400
200
0
a
b
c
a
Control
RA
RA+BMP
RA+activin

Figure 3.8. The additive effect of RA and FS on the *Nkx2.5* expression RA and follistatin have an additive effect in inhibiting *Nkx2.5* expression. *Nkx2.5* expression is not significantly inhibited with low levels of RA (0.1 μM) or FS (5 ng/ml) compared with high levels of them (not shown here), but the inhibitory effect is obviously enhanced when both were added together. The same letters indicate no differences statistically. n value in box indicates explant number for measurement.


Average area of Nkx2.5 expression domain (Pixel)
1800
1600
1400
1200
1000
800
600
400
200
0
a
b
b
c
10
10
10
10
Control
FS
RA
FS+RA

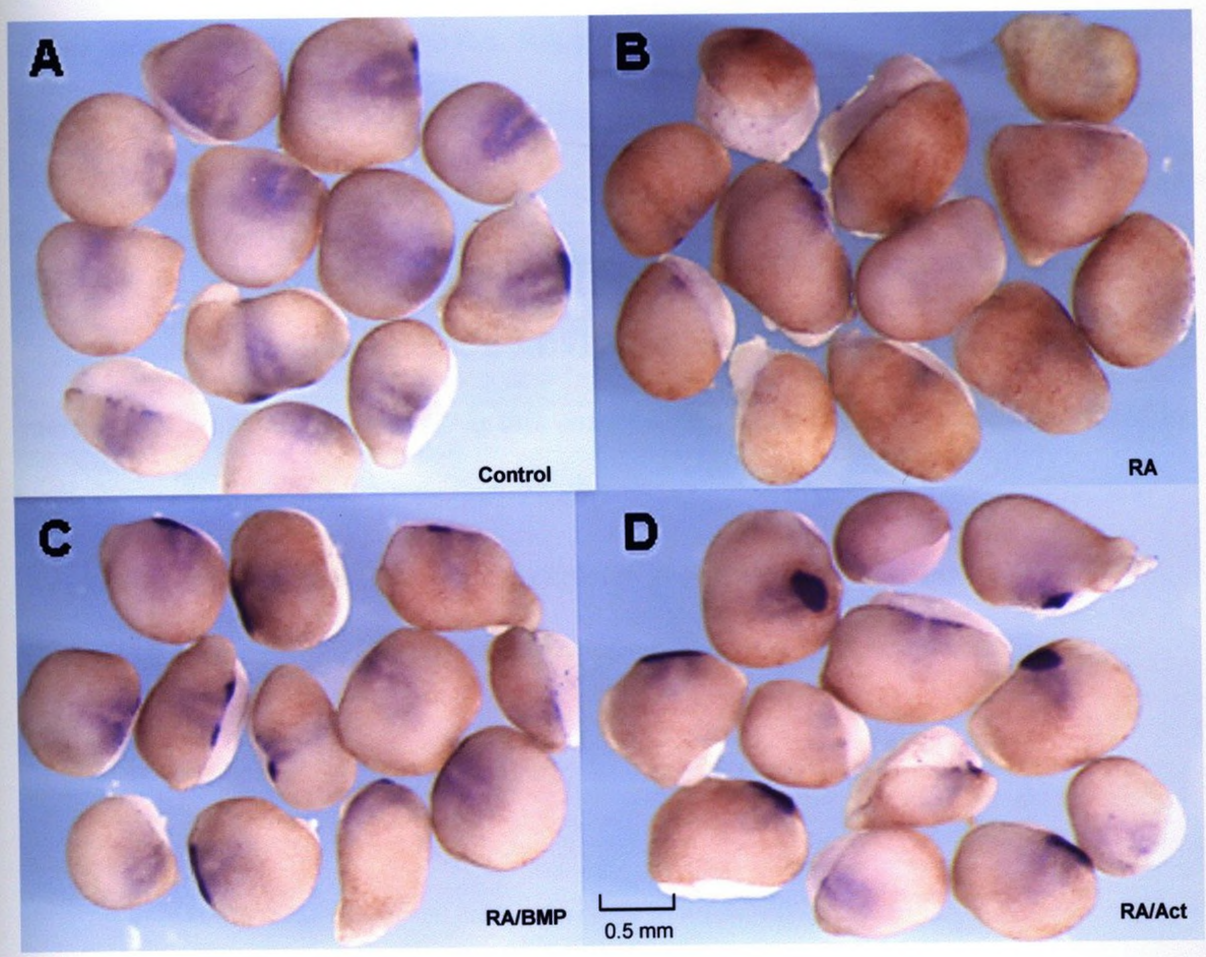
experiment as discussed above for Nkx2.5. When BMP is added, the inhibitory effect of excess RA is blocked in cultured explants (Fig. 3.9). These results suggest that RA represses *Tbx20* gene expression through decreasing BMP signaling. To our knowledge, this is the first report that RA regulates *Tbx20* expression and that it does so in a BMP dependent manner.

Our previous experiments have shown that activin rescues the inhibitory effect of RA and follistatin on *Nkx2.5* expression. We also performed blocking experiments by activin for Tbx20. Our results showed that activin can rescue RA-induced inhibition of *Tbx20* expression (Fig. 3.9), but the blocking effect is not as significant as activin does to *Nkx2.5* (Fig. 3.7).

3.7 The effects of excess RA on BMP gene expression

Our rescue experiments indicated that a decreased BMP activity is responsible for the inhibition of cardiomyocyte differentiation by RA. In order to clarify the mechanism for decreased BMP signaling, we examined the effects of RA on BMP expression since decreased expression of BMPs may lead to a reduction of BMP signaling. We wished to test if the reduced levels of BMP signaling could correlate with reduced expression of *BMP2, 4* and *7*. These BMPs are all expressed in the developing myocardium, so we examined the changes of *BMP* 2, 4 and 7 expression domain caused by addition of excess RA and RA antagonist. Our results showed that neither excess RA nor deficient RAnoticeably affects expression domain of *BMP*2, 4 and 7 (Fig. 3.10). As whole mount *in situ* hybridization does not allow for precisely quantitative measurements of gene expression, it remains possible that alterations in expression level exist. However, these

Figure 3.9. BMP rescues RA's effects on the *Tbx20* expression. BMP can reverse the inhibitory effect of RA on *Tbx20* expression. When compared to control explants of the heart region (A), addition of RA reduces the size of the expression domain (B). In the presence of BMP (C) or activin (D), *Tbx20* expression domain increases compared with only RA treated explants (B).

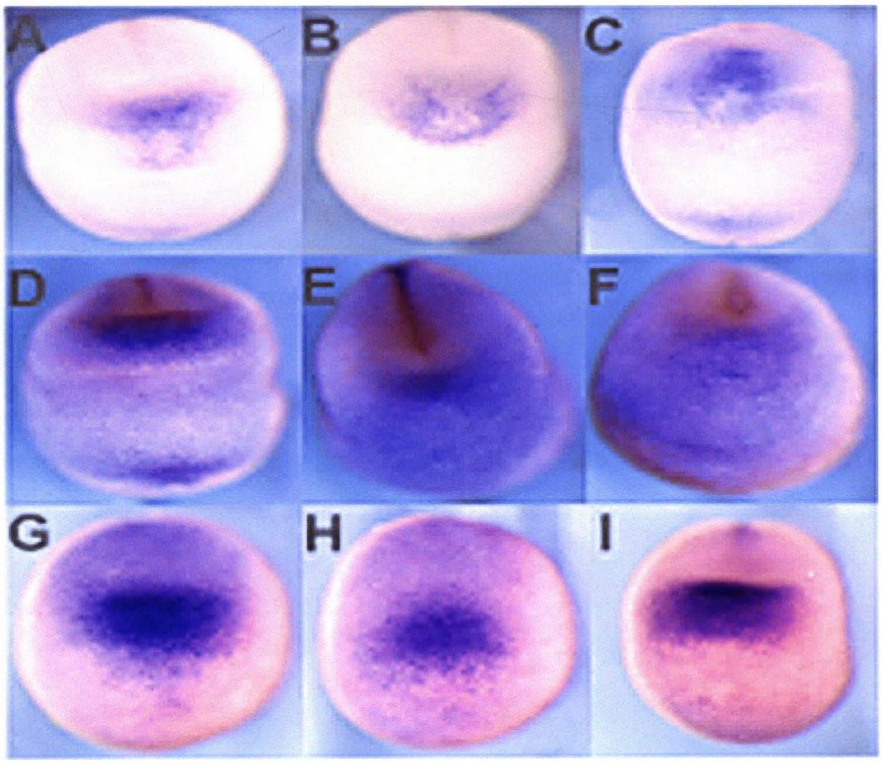

A
Control
B
RA
C
RA/BMP
D
RA/Act
0.5 mm

results suggest that any change is likely small and would not likely account for the changes observed in cardiac differentiation.

3.8 The effects of the endogenous RA on *Nkx2.5* gene expression

In all of the above experiments, RA was added to the media and thus all of cells in the explants were exposed to the RA. RA synthesis is localized to the posterior end of the heart tube and that raises the possibility that the exogenous RA is only having an effect due to the cardiomyocytes being exposed to levels of RA that they would never normally encounter. Therefore, we examined the effects of increasing endogenous RA on the heart using ketoconazole. Ketoconazole is a CYP26 inhibitor, the enzyme responsible for metabolizing RA, and by inhibiting this enzyme, endogenous RA levels are increased in areas where it is normally expressed. Our results showed that *Nkx2.5* gene expression was inhibited in ketoconazole-treated explants in a concentration dependent manner (Fig.3.11). These results indicate that increasing endogenous RA can inhibit cardiogenesis.

Figure 3.10. The effects of RA and RAA on *BMP2, 4* and *7* gene expression. RA does not affect the domain of *BMP2* (A-C), *BMP4* (D-F) and *BMP7* (G-I) expression. Embryos at stage 14 were treated with RA (1 μM) (C, F, I), RAA (1 μM) (A, D, G) and DMSO as control (B, E, H) and were allowed to develop until stage 20 for whole mount *in situ* analysis. Embryos are oriented with ventral views and anterior to the top.



BMP2

BMP4

BMP7

RAA **DMSO** **RA**

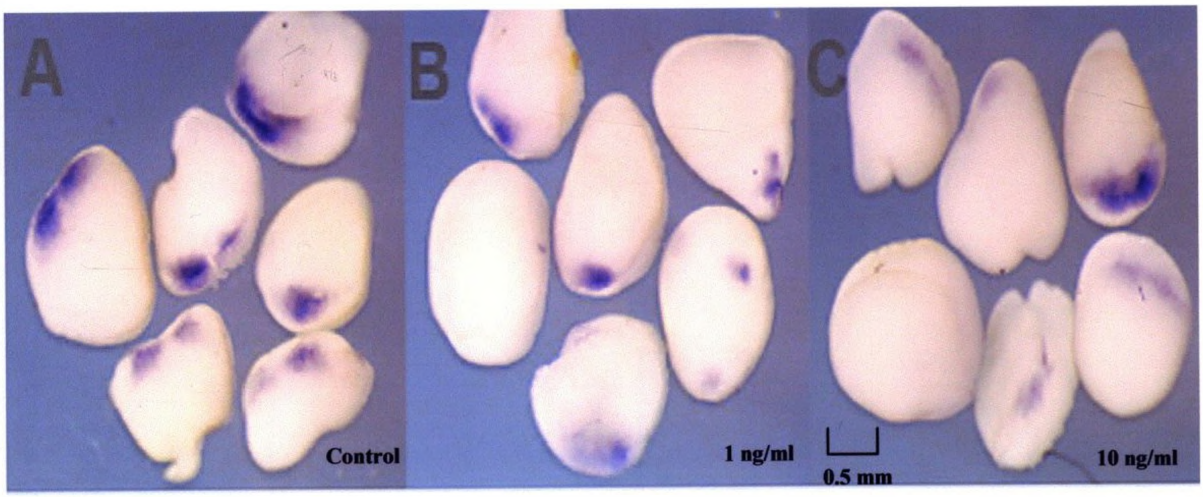
Figure 3.11. The effect of endogenously increased RA on *Nkx2.5* gene expression

I, When compared to control explants of the heart region (A), addition of ketoconazole reduces the size of the expression domain (B). The higher concentration of ketoconazole (10 ng/ml) (C) has more potent inhibitory action on *Nkx2.5* gene expression than the lower concentration (1 ng/ml) (B).

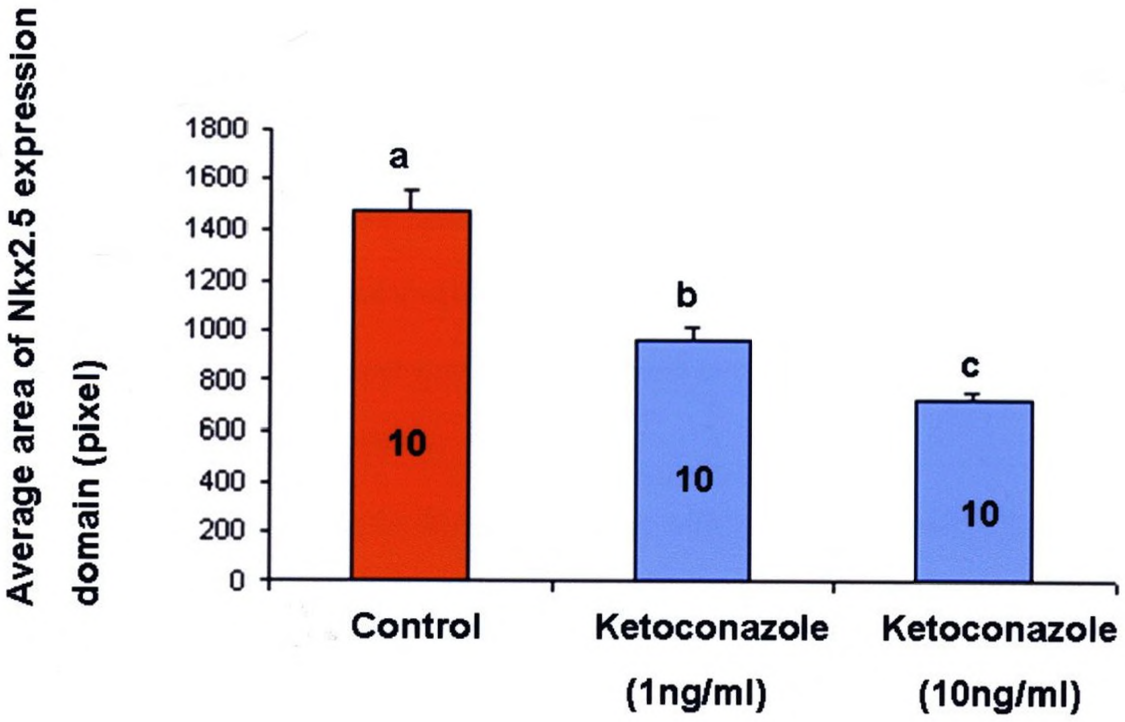
II, Quantified results are shown.

The same letters indicate no statistical differences. n value in box indicates number of explants for measurement.

I



II


Average area of Nkx2.5 expression domain (pixel)
1800
1600
1400
1200
1000
800
600
400
200
0
a
b
c
10
10
10
Control
Ketoconazole (1ng/ml)
Ketoconazole (10ng/ml)

CHAPTER 4 DISCUSSION

In vertebrates, the heart is the first organ to form. Heart development is a beautifully orchestrated process that is highly conserved throughout evolution. The heart of all vertebrates is practically indistinguishable at the linear heart tube stage and into the early stages of looping morphogenesis and chamber formation.

RA is an important signaling molecule that plays critical roles in the regulation of the heart development (Zile, 2004). RA can affect all of the early signaling molecules regulating cardiac progenitor differentiation, including Wnts, FGFs and BMPs (Zile, 2004). RA deficiencies lead to widespread cardiac defects (Zile 2004; Collop et al., 2006; Xavier-Neto et al., 2001) and excess RA also inhibits cardiomyocyte differentiation in *Xenopus* (Drysdale et al., 1997).

In order to investigate the mechanism by which excess RA inhibits cardiomyocyte differentiation, Affymetrix GeneChip® technology had previously been performed in our Lab to examine global gene expression changes induced by addition of RA and RA antagonist. Two of the genes that had altered expression were *follistatin* and *Tbx20*. Follistatin is an inhibitor of BMP and increased expression of *follistatin* can decrease BMP signaling. Tbx20 is an important cardiac transcription factor and plays a crucial role in the regulation of heart development. Due to the importance of the signaling molecules in development, I chose to specifically assess their importance on heart development.

It is known that BMP signaling is a required for cardiomyocyte differentiation (Monzen et al., 2001; Syi et al., 2002; Ladd et al., 1998 and Olson et al., 2006). Therefore, we developed a hypothesis that the increased *follistatin* expression by RA may be responsible for the inhibition of the myocardial differentiation through opposing

BMP activity.

4.1 RA inhibits cardiomyocyte differentiation through decreasing BMP signaling

Several lines of evidence demonstrate that BMP signaling is required for cardiogenesis. BMPs are expressed in the heart-forming region during cardiac formation (Schultheiss et al., 1997; Shi et al., 2000). In *Drosophila*, loss of decapentaplegic, the homologue of BMP in fly, results in a loss of the heart (Yin et al., 1998). In vertebrates, application of BMP inhibitors such as noggin blocks heart differentiation (Shi et al., 2000). A major function for BMP in cardiogenesis is to maintain the expression of *Nkx2.5* (Akazawa and Komuro 2005) and this in turn is necessary for full expression of cardiac differentiation genes. Addition of RA has been shown to significantly inhibit *Nkx2.5* expression (Drysdale et al., 1997; Vamali et al., 2001).

Our experiments clearly demonstrate that addition of BMP4 or activin can rescue the inhibitory effects of excess RA on *Nkx2.5* expression (Fig. 3.5). Therefore, in the heart, disruption of the BMP signaling system is one major consequence of RA addition. There are multiple levels where BMP signaling can be attenuated. Transcriptional regulation of either the ligand or receptor could be a possibility. However, our *in situ* results showed that excess or deficient RA does not affect BMP expression at least at the level of transcription (Fig. 3.10). Although the whole mount *in situ* hybridization is not quantitative, the lack of substantial changes suggests that the loss of BMP signaling is not due to changes in expression of *BMP*.

We did not test for alterations in the presence of the BMP receptors. There is recent evidence that regulation of BMP receptor levels is a mechanism used to

suppress BMP signaling during development of the *Xenopus* nervous system, however, expression of dullard, the protein required for targeted destruction of the BMP receptor, is not expressed near the heart. This still leaves the possibility that transcriptional regulation of the BMP receptors may play a role in the effects of RA on the heart.

Importantly, excess activin or BMP can rescue the inhibitory effect of RA on cardiogenesis (Fig. 3.17), suggesting that follistatin's effects are at the levels of binding the receptors. There are several possible mechanisms that may be involved in the rescue effect of activin on the cardiac differentiation. Firstly, activin is a strong antagonist of follistatin which can inhibit BMP signaling. Chick explant experiments have demonstrated that addition of activin promotes cardiogenesis and the BMP inhibitor, noggin, can block the strengthening effect of activin on BMP, which indicates that addition of activin enhances BMP activity (Ladd et al., 1998; Logan and Mohun 1993). Our experiments have shown that RA increases follistatin expression in the heart, therefore, I suggest that activin can indirectly strengthen BMP signaling through opposing follistatin. The antagonism of activin on follistatin may explain the ability of activin to strengthen BMP signaling in the chick.

Secondly, activin may have a direct inductive role in the regulation of heart development. In *Xenopus*, the animal caps can be induced into beating heart by treating with activin (100 ng/ml) (Mohun et al., 1993). However, the mechanism for its direct inductive role is currently unknown.

Alternatively, activin can significantly enhance expression of both Wnt11 and its receptor Fz-7 and both are required for heart development (Pandur et al., 2002). Therefore, Wnt11 and its receptor may be involved in activin-induced antagonism of the

inhibition by RA. In early development, activin is expressed in the brain, eye enlagen, visceral pouches, otic vesicals, and the anterior notochord, but it is not expressed in the heart-forming region in *Xenopus*. Based on these findings, I concluded that activin does not directly activate the cardiogenic programme at the stages we used for our experiments.

4.2 Follistatin is involved in RA-induced inhibition of cardiomyocyte differentiation

I have confirmed that follistatin expression increases in response to RA treatment. As follistatin is a secreted protein that plays an important role during development by antagonizing both BMP and activin (Hemmati-Brivanlou et al., 1994), I decided to investigate whether follistatin might play a role in altering the differentiation of the heart. Follistain is first produced in the organizer along with noggin and chordin and is involved in the neural formation by opposing BMP activity (Hemmati-Brivanlou et al., 1994). BMP is also a ventralizing factor during formation and dorsal-ventral patterning of the mesoderm. Mesoderm exposed to BMP takes a ventral mesoderm fate such as kidney or blood islands, while suppression of BMP signaling by BMP antagonists such follistatin, noggin, and chordin allows for differentiation of dorsal mesoderm derivatives. A similar system is used in differentiation of the ectoderm where neural development results when BMP is antagonized (Hemmat-Brivanlou et al., 1994).

BMP signaling is often blocked by inactivation of the secreted BMP ligands by other secreted proteins that bind and either target BMPs for degradation or prevent them from interacting with their receptor.Therefore, the potential role of increased follistatin as a potential mechanism for the loss of *Nkx2.5* and *Tbx20* expression was examined. Addition of follistatin inhibits the size of the *Nkx2.5* expression domain

(Fig 3.5) indicating that this is indeed a possibility. There are a number of other known BMP antagonists that could inhibit BMP signaling if they were up-regulated by addition of RA such as noggin. Noggin is a BMP inhibitor and can antagonize BMP signaling in many developmental contexts. Two forms of *noggin* have been described in *Xenopus.* In the early embryo noggin1 is expressed beginning in the blastula organizer region and continuing through gastrulation and neurulation in the organizer and notochord. Later, it is also expressed in the anterior neural ridge and subsequent forebrain (Fletcher et al., 2004). At the tadpole stages expression is maintained in the dorsal neural tube and is present in the otic vesicle. The expression of noggin2 is similar to the expression of noggin1 except that it is expressed in the heart by stage 28. Recent preliminary experiments indicated that excess or deficient RA does not alter noggin expression (Drysdale, unpublished). Therefore, in *Xenopus,* it is unlikely that noggin plays a role in suppressing BMP activity during early heart development.

Additionally, *follistatin* is expressed near the posterior end of the heart tube prior to overt cardiac differentiation in normal embryos and this expression is greatly increased by the addition of retinoic acid (Fig. 3.1). Most importantly, while addition of BMP could rescue the expression of *Nkx2.5*, the addition of activin, which specifically binds to follistatin, was able to rescue the effects of RA (Fig 3.7). This suggests that the over expression of *follistatin* rather than another BMP inhibitor is responsible for the loss of *Nkx2.5* expression.

Our results showed that excess RA markedly increases *follistatin* expression and follistatin can inhibit cardiomyocyte differentiation (Fig. 3.1 and 3.5). The inhibitory effects of follistatin can be rescued by BMP4 and activin, which indicates that follistatin

inhibits cardiogenesis through decreasing BMP signaling rather than an unrelated mechanism (Fig. 3.5). Since activin specifically antagonizes follistatin, the ability of activin to rescue the inhibitory effects of both excess RA and follistatin further demonstrates that follistatin is likely involved in RA-induced inhibition (Ladd et al., 1998).

To determine if RA and follistatin are on the same signaling pathway, I examined the additive effects of RA and FS on *Nkx2.5* expression. Low levels of RA or FS inhibit *Nkx2.5* expression to a significant level. However, the combination of FS and RA results in additive effect in the inhibition of *Nkx2.5* expression (Fig. 3.8). Since the effects are additive, it further indicates that FS and RA are working in a linear pathway.

4.3 Is follistatin required for normal heart development?

The developmental profile of follistatin shows that follistatin is expressed in the ventral side below the cement gland from stage 20 to stage 32, suggesting one possible role of follistatin on determining D-V patterning. The expression of *follistatin* overlaps at least partially with *Nkx2.5, Tbx20* and *cTnI* (Fig. 3.3 and 3.4).

In *Xenopus*, recent studies revealed that the depletion of *chordin*, *noggin* and *follistatin* results in a catastrophic loss of dorsal development, with excessive amounts of ventral tissues at the expense of dorsal structures (Khokha et al., 2005). Inactivation of two of these BMP antagonists has only a weak effect. Similarly in the mouse, double inactivation of *chordin* and *noggin* affects only the development of prosencephalon. Therefore, *follistatin* may be responsible for dictating specific heart regions.

Overexpression of BMP may be responsible for congenital heart diseases and

finely regulated BMP levels are necessary for normal heart development (Prall et al., 2007). Alternatively, *follistatin* may be affecting cardiomyocyte proliferation. In mouse, noggin has been shown to regulate cardiomyocyte proliferation *in vivo* (Choi et al., 2007). Removal of noggin causes increased BMP signaling in the heart. The higher level of BMP signaling in the myocardium causes increased proliferation and this phenotype was rescued by reduction of *BMP4*, indicating that BMP4 is at least one of the relevant BMP ligands antagonized by noggin in the control of myocardial proliferation. This fits well with the observation that *BMPR1a* loss reduces myocardial proliferation because BMP4 signals are transduced by BMPR1A (Mishina et al., 2003).

However, in *Xenopus,* neither noggin1 nor noggin2 is expressed in the heart prior to stage 28, so we do not think that noggin is involved in balancing BMP signaling at the early stages we are examining. This does indicate that regulating the intensity of BMP signaling is necessary for normal myocardial proliferation and provides one potential role for follistatin in normal heart development.

Currently, I cannot conclude what the normal role for follistatin is during heart development. However, based on the expression of follistatin at the correct time and place and our demonstrated activity of follistatin on the developing heart, I conclude that follistatin is playing a role in cardiogenesis, likely by regulating BMP activity.

4.4 RA inhibits Tbx20 expression in heart-forming region

Tbx20 is an the important cardiac transcription factor and is involved in early heart development. In *Xenopus*, injection of antisense morpholinos to *Tbx20* caused reduced cardiac mass (Brown et al., 2005). In mice, *Tbx20* mutants also show hypoplasia

of the future right ventricle and lack a morphologically distinct outflow tract (Takeuchi et al., 2005). These results demonstrate that Tbx20 plays a crucial role in cardiogenesis.

Our results showed that excess RA inhibits *Tbx20* expression in the heart-forming region (Fig. 3.2) and provides another mechanism by which excess RA inhibits cardiogenesis. It is unclear if RA inhibits Nkx2.5 and Tbx20 in the same pathway. My results show that BMP4 can rescue inhibition of both *Tbx20* and *Nkx2.5* expression by RA, suggesting this possibility. However, the further investigation is needed to determine if these are separate events or whether a common mechanism is used to inhibit both Tbx20 and Nkx2.5.

4.5 Endogenous RA is important to heart formation

Most of the time exogenous RA is used to investigate the effects of RA on heart development (Collop et al., 2006). Although RA can alter cardiac development, it is not normally made there. To address this concern, we used ketoconazole to alter RA levels by inhibiting CYP26, which is responsible for RA degradation.

The *Nkx2.5* expression domain was decreased in ketoconazole-treated culture explants compared with the control (Fig. 3.11) and the inhibitory effects of ketoconazole increase at higher ketoconazole concentrations. These findings indicate that the inhibition of *Nkx2.5* expression can occur at physiological levels of RA, suggesting that endogenous RA plays an important role in regulating heart formation. In addition, both administration of exogenous RA and endogenously increased levels show similar ability in inhibiting *Nkx2.5* gene expression. Therefore, it appears that precise control over the levels of RA is ecessary for normal heart development. This indicates that the administration of

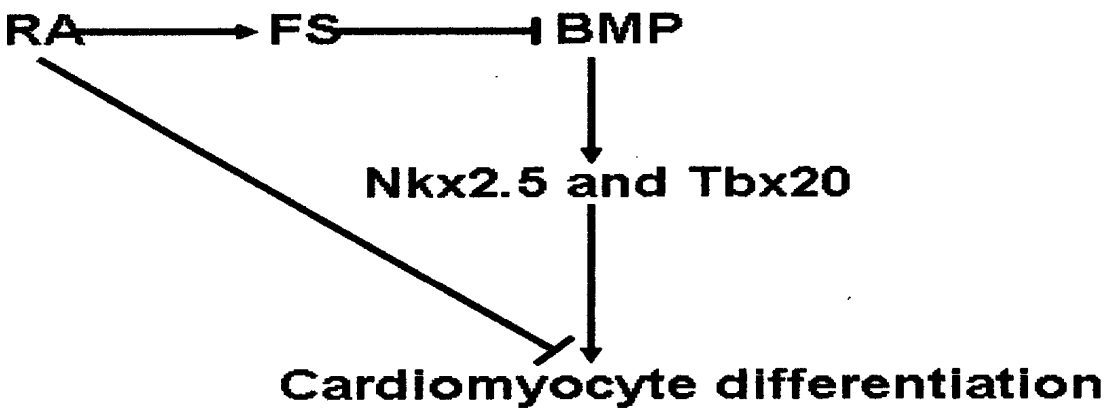
exogenous RA can provide clues to investigate physiological functions of RA.

In summary, these results suggest that excess RA inhibits cardiogenesis through decreasing BMP signaling. The inhibition of heart development and *Nkx2.5* expression by follistatin is rescued by increased activin, a follistatin inhibitor. This indicates that excessive RA induces *follistatin* expression and inhibits *Tbx20* expression, providing a mechanistic pathway for the inhibition of heart development. Based on this data, I have proposed a working model of RA's inhibitory effects on cardiomyocyte differentiation (Fig. 4.1).

4. 6 Conclusions

1) RA inhibits *Nkx2.5* gene expression through decreasing BMP signaling since BMP can rescue the inhibitory effects of RA. Activin rescues the inhibitory effect of RA by strengthening BMP signaling.
2) The expression of *follistatin* is sensitive to RA levels and excess RA increases *FS* gene expression while RA antagonist decreases its expression.
3 Follistatin can inhibit *Nkx2.5* expression and its inhibitory effect can be rescued by BMP4 and activin.
4 *Follistatin* is expressed in the heart-forming region during the early heart development, suggesting that *follistatin* is involved in the heart development.
5 RA inhibits *Tbx20* gene expression through decreasing BMP signaling as BMP4 rescues this inhibitory effect.
6 Endogenously increased RA inhibits *Nkx2.5* gene expression, suggesting that RA is required for cardiogenesis.

Figure 4.1. A model of the effects of RA on cardiomyocyte differentiation RA inhibits heart development through follistatin. The increases *follistatin* expression follistatin inhibits BMP signaling which is required for the maintenance of *Nkx2.5* and *Tbx20* expression. The decreased BMP signaling reduces *Nkx2.5* and *Tbx20* expression which is essential for normal heart development.


RA
FS
BMP
Nkx2.5 and Tbx20
Cardiomyocyte differentiation

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