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RETINOIC ACID REGULATION OF CARDIAC INFLOW TRACT DEVELOPMENT IN THE AVIAN EMBRYO IS MEDIATED VIA A TGFbeta2 SIGNALING PATHWAY.

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RETINOIC ACID REGULATION OF CARDIAC INFLOW TRACT DEVELOPMENT IN THE AVIAN EMBRYO IS MEDIATED VIA A TGFβ2 SIGNALING PATHWAY

By

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ABSTRACT

RETINOIC ACID REGULATION OF CARDIAC INFLOW TRACT DEVELOPMENT IN THE AVIAN EMBRYO IS MEDIATED VIA A TGFβ2 SIGNALING PATHWAY

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Vitamin A is essential for normal embryonic cardiac morphogenesis. The vitamin A-deficient (VAD) phenotype in the avian embryo includes an abnormal heart-tube closed at the inflow tracts and the absence of omphalomesenteric veins that normally connect the embryo to the extraembryonic circulatory system. In VAD embryos, there were decreased presumptive endothelial cells in the heart-forming region and in the area pellucida from the beginning of differentiation (1 ss), through the initiation of heart beating (10/11 ss). TGFβ2, a multi-functional growth regulator and TGFβ2 mRNA were increased in the posterior heart-forming region of VAD embryos. Significantly, TGFβ2 transcripts were increased in the inflow tracts and the adjacent head mesenchyme prior to inflow tract closure in the VAD embryo. The administration of TGFβ2 antisense oligonucleotides to the VAD embryo, specifically decreased TGFβ2 and TBRII transcripts in the inflow tracts, which remained open, and increased presumptive endothelial cells were observed in the inflow tracts and the surrounding area pellucida. Our results indicate that TGFβ2 is a component of the retinoid-mediated cardiogenic pathway, specific for inflow tract development.

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KEY TO ABBREVIATIONS

Ab	antibody
atRA	all-trans-retinoic acid
atROH	all-trans-retinol
BLAST	Basic Local Alignment Search Tool
CRABP	cellular retinoic acid binding protein
ECM	extracellular matrix
EN	endocardium
EP	epimyocardium
FITC	fluorescein isothiocyanate
FN	fibronectin
HFR	heart-forming region
HH	Hamburger and Hamilton (stages of avian embryonic development)
HM	head mesenchyme
Hn	Hensen's node
IFT	inflow tract
LAP	latency associated protein (TGFβ)
LRAT	lecithin:retinol acyltransferase
LTBP	latent TGFβ binding protein
MoAb	monoclonal antibody
NT	neural tube
PFA	paraformaldehyde

PBS	phosphate buffered saline
RA	retinoic acid
RAE	retinol activity equivalents
RAR	retinoic acid receptor
RARE	retinoic acid responsive element
RE	retinyl ester
RBP	retinol binding protein
RXR	retinoid X-receptor
ss	somite stage
TBR	TGFβ receptor
TGFβ	transforming growth factor beta
TRITC	tetramethylrhodamine isothiocyanate
TTR	transthyretin
VAD	vitamin A-deficient
VEGF	vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Vitamin A: historical background

Night blindness was a recognized disease entity in ancient Egypt and it was suggested that a component in food was essential for night vision (McLaren, 1980). McCollum and Davis (1913) discovered a fat-soluble component of food that was capable of promoting growth in rats. This substance was initially termed "Fat Soluble A" and was later named vitamin A. Early researchers focused on physiology and metabolism of vitamin A. Wald (1934) and Morton (1944) determined that the chromophore of the visual pigment is retinaldehyde. These early studies elucidated the role of vitamin A in vision (Wald, 1968) and confirmed the ancient Egyptian beliefs about essential components in food.

In 1937 Holmes and Corbet (1937) crystallized pure retinol from fish liver. Later, Arens and van Dorp (1946) and Isler and his associates (Isler et al., 1947) succeeded in achieving the chemical synthesis of pure retinoic acid and retinol. These discoveries provided the groundwork on which further research was based to study the function, metabolism, regulation, and pharmacological use of vitamin A.

The importance of vitamin A throughout the life cycle has been well established. The requirement for vitamin A begins early in normal embryonic development (Wolf, 1984; Hofmann and Eichele, 1994) and continues in the adult organism (Moore, 1957; DeLuca, 1991; Gudas et al., 1994; DeLuca et al.,

1997). Although it was clear that vitamin A was essential, the mechanism of action used in vision could not explain the vast biological processes that were regulated by vitamin A. Many researchers focused on the molecular mechanism of action of vitamin A and these mechanisms are still under investigation. The regulation at the gene level attributed to vitamin A is due to all-trans-retinoic acid (atRA), the transcriptionally active form of vitamin A and a recognized regulator of cell division and differentiation in tissues of ectodermal, endodermal, and mesodermal origin (Roberts and Sporn, 1984; Wolf, 1984; Gudas et al., 1994). Recent advances in the understanding of the mechanisms of action of vitamin A can be attributed to the discovery of nuclear receptors for retinoic acid (RARs) and their nuclear receptor partners, the retinoid X-receptors (RXRs)(Mangelsdorf et al., 1995; Pfahl and Chytil, 1996). In light of the discoveries about the molecular mechanism, the term "retinoids" has recently been redefined to refer to any substance that exerts vitamin A function via retinoic acid nuclear receptors, eliciting a biological response (Sporn and Roberts, 1994).

Vitamin A and more generally, the retinoids, are capable of producing alterations in the expression of a diverse group of genes (DeLuca, 1991; Gudas et al., 1994). The discovery of the retinoid specific nuclear receptors provided an explanation as to how vitamin A controls such a large network of gene activation processes. Genes that have retinoic acid response elements (RARE) in their promoter region are affected by vitamin A status and are involved in diverse

biological functions; however, their functions are interconnected (DeLuca, 1991; Mangelsdorf et al., 1995; Mangelsdorf et al., 1996).

Inadequate maternal vitamin A during pregnancy results in fetal death and congenital malformations (Mason, 1935; Hale, 1937). Vitamin A deficiency affects several target tissues including the heart, the ocular tissues, and the circulatory, urogenital, respiratory and central nervous systems (Wilson et al., 1953; Thompson, 1969; Zile, 1998; Gale et al., 1999; Maden, 1999; Zile, 1999; Ross et al., 2000). At the other end of the spectrum, excess vitamin A causes congenital malformations during embryogenesis and is hepatotoxic in adult animals (Nau et al., 1998). The targets of the teratogenic effects of excess retinoids have been demonstrated to be the heart, skull, skeleton, limbs, central nervous system, brain, eyes, and craniofacial structures (Moore, 1957; Morriss and Steele, 1972; Shenefelt, 1972; Brockes, 1989; Kochhar et al., 1993; Rosa, 1993; Kochhar and Christian, 1997; Nau et al., 1998;). There is considerable overlap in the targets and the malformation associated with vitamin A deficiency and with exposure to excess retinoids. These findings suggest that there are common targets in deficiency and in excess and that retinoid regulation is required for proper development and health.

1.2 Vitamin A and human nutrition

As elucidated in the previous section, vitamin A is an essential nutrient. Vitamin A enters the body through consumption of foods that contain preformed dietary vitamin A, or provitamin A carotenoids. The absorption, metabolism and

transport of retinoids has most recently been reviewed by Blaner et al. (1999). Retinoids are converted to retinol (ROH) and solubilized into mixed-micellar solution before efficient absorption can occur at the brush boarder of the small intestine. ROH is converted to retinyl ester (RE), through the action of the intestinal lecithin:retinol acyltransferase (LRAT) for packaging in nascent chylomicrons (Quick and Ong, 1990; Ong et al., 1994). Lipoprotein lipase catalyzes lipolysis of nascent chylomicrons in the lymphatic system, giving rise to chylomicron remnants, which enter the circulation and are transported to the liver for storage. RE of chylomicron remnants are first taken up by parenchymal cells of the liver and are then transferred to stellate cells for storage. The stellate cells contain enzymes that can hydrolyze RE to ROH when ROH is needed. Subsequently in hepatocytes, ROH is bound to the retinol binding protein (RBP)-transthyretin (TTR) complex for transport in the plasma to the target tissues. ROH is taken-up by the target cells. The exact mechanism for cellular uptake of retinol is still unclear. It is possible that there is more than one mechanism for cellular-uptake, which may be mediated by the cell type and retinoid status in the target cells. Inside the cell retinol can be metabolized; specific proteins bind retinol and direct it to retinoid-metabolizing enzymes (Napoli, 2000). These proteins act as chaperones helping retinol to be metabolically converted to retinoic acid (RA), the transcriptionally active form of vitamin A (McCaffery and Drager, 1994; Chen et al., 1995; Niederreither et al., 1997; Moss et al., 1998). RA is transported to the nucleus where it acts via its nuclear receptors,

the RARs and RXRs, to control transcription of a diverse group of genes (Gudas et al., 1994).

A small percentage of dietary provitamin A and preformed vitamin A is converted to RA in the intestine. RA is absorbed in the intestine via the portal system. In circulation it is bound to serum albumin (Blaner and Olson, 1994) and is delivered to target cells, where uncharged RA rapidly and spontaneously crosses the cell membrane (Noy, 1992). Once inside the cell, RA binds to either cellular retinoic acid-binding protein I or II (CRABP) in the cytoplasm, which acts as a chaperone, protecting the molecule until it is used in the nucleus (Chytil and Ong, 1983; Napoli et al., 1991). While there is metabolic interconversion between the reduced forms of vitamin A, RA cannot be reduced and converted to ROH.

Vitamin A in the diet comes from preformed retinoids as well as provitamin A carotenoids. Preformed vitamin A is present in meat and dairy products, the best source being liver. Many fish liver oils may contain up to 300 μg of vitamin A per gram of oil (Moore, 1957; Lui and Roels, 1980). Unlike preformed retinoids, provitamin A carotenoids are found in plant sources. There are more than 600 carotenoids found in nature; however, only approximately 50 can be converted to vitamin A (Blaner and Olson, 1994). Carotenoids are widely distributed in colored fruits and vegetables. Carrots are the major dietary source of α- and β-carotene (Chug-Ahuja et al., 1993). The Continuing Survey of Food Intakes by Individuals (CFSII) in 1994-1996 reported that grains and vegetables were the major source of vitamin A in the diet (approximately 55 percent),

followed by dairy and meat products (approximately 30 percent). The bioconversion of carotenoids and the associated ROH activity is still under scrutiny (Hume and Krebs, 1949; Torronen et al., 1996; Castenmiller and West, 1998; Rock et al., 1998; Van den Berg and van Vliet, 1998; Boileau et al., 1999). Retinol Equivalent units were implemented to provide a common unit of measurement for preformed retinoids and carotenoids based on observed ROH activity (NRC, 1980; 1989). However, the ratios used to determine Retinol Equivalents were not universally accepted, and Retinol Activity Equivalents (RAE) replaced Retinol Equivalents in the 2001 Dietary Reference Intakes (DRI) (NRC, 2001). RAE, like their predecessors, the Retinol Equivalents, provide ratios to convert provitamin A carotenoids into a common unit of measure based on ROH activity of carotenoids. Using RAE, 12 μg β-carotene is considered to have the activity of 1 μg of ROH (Hume and Krebs, 1949; Chug-Ahuja et al., 1993; Parker et al., 1999). Other dietary carotenoids have a conversion ratio of 24:1 (μg carotenoid: μg ROH) based on the observation that the activity of other carotenoids is approximately half that of β-carotene (Deuel et al., 1949; Bauernfeind, 1972). The vitamin A activity of provitamin A carotenoids using RAE is half the vitamin A activity when using Retinol Equivalents and can have a substantial impact on assessing vitamin A nutriture since a large portion of vitamin A in the diet is obtained through the consumption of carotenoid rich fruits and vegetables.

Using RAE for conversion of carotenoids to vitamin A, the DRI for vitamin A is set at 900 μg for men and 700 μg for women (NRC, 2001).

Absorption of vitamin A in the small intestine is affected by dietary fat, infection, food matrix and processing, as well as nutrient-nutrient interactions with iron, zinc, and alcohol (Dorea and Olson, 1986; Suharno et al., 1993; Jalal et al., 1998; Van het Hof et al., 1998; Boileau et al., 1999; Wang, 1999; Tang et al., 2000). These factors and others need to be considered when making individual recommendations and assessing nutriture based on consumption.

1.3 Vitamin A deficiency

There is an intense interest in vitamin A in biology and in medicine, due to the essentiality of vitamin A throughout life. It is well established that maternal insufficiency of vitamin A results in death of the fetus as well as congenital malformations, including those associated with the heart (Wolf, 1984; Blomhoff, 1994; Zile, 1998; Ross, 2000). Vitamin A is also required throughout life for maintenance of differentiation, proliferation, and apoptosis (Gudas et al., 1994), as well as playing a critical role in reproduction and vision (Wald, 1968).

Xerophthalmia is synonymous with all of the clinical signs and symptoms that affect the eye in vitamin A deficiency and is the most common clinical effect of inadequate vitamin A intake (McLaren and Frigg, 2001). Some of the common signs and symptoms of xerophthalmia are: night blindness, conjunctival xerosis, and Bitot's spot. There are an estimated 3 to 10 million children who become xerophthalmic and 250,000 to 500,000 who become blind annually, mostly occurring in developing countries (WHO, 1995; Sommer and West, 1996;). Xerophthalmia is the most common cause of blindness in the world. In

addition to the cases of clinical deficiency, it is estimated that there are 251 million children with subclinical vitamin A deficiency (WHO, 1995). Subclinical vitamin A deficiency has been associated with increased morbidity and mortality in children (Beaton et al., 1994; Sommer and West, 1996). Many children with subclinical vitamin A deficiency will go undiagnosed, increasing their risk for morbidity and mortality, due to the inadequacy of many of the health care systems in the developing countries that have a high prevalence of vitamin A deficiency. This problem is complicated by the fact that the methods used to assess vitamin A status are not adequate due to their lack of sensitivity and practicality (Rosales and Ross, 1998; Filteau et al., 2000). It is important to note that children are not the only ones subject to vitamin A deficiency; however, children in developing countries are at the greatest risk.

The adverse affects associated with vitamin A deficiency make determining vitamin A status essential, especially when dealing with high-risk groups. Five states of vitamin A nutriture have been identified for defining vitamin A status: deficient, marginal, satisfactory, excessive, and toxic (Olson, 1994). Vitamin A is stored in the liver as RE and the amount of vitamin A stored in the liver is the key determinant when assessing vitamin A status. Various methods have been developed to assess vitamin A status, each with strengths and weaknesses; however, the methods currently available are insufficient to properly assess vitamin A status (NRC, 2001). Assessment of vitamin A status by plasma vitamin A and RBP levels is not a valid method, because plasma levels of vitamin A, in the form of retinol bound to RBP and TTR, are

maintained under tight homeostatic control and are relatively unaffected by vitamin A status, except when liver stores are very low or very high (McLaren and Frigg, 2001). In addition to the lack of sensitivity of plasma values to reflect actual liver reserves, plasma values are also negatively influenced by the presence of infection and protein-energy malnutrition (Filteau et al., 2000), rendering them ineffective for measuring vitamin A status; however, they are effective for population-based studies. Liver biopsy is the only accurate measure of vitamin A status over all five states of nutriture; however, the invasive nature and the expense of the procedure makes it less than ideal for assessing vitamin A status, especially with regards to population-based studies. The methods currently available to test vitamin A status are inadequate, especially with regards to marginal status. Researchers are working to improve the methods for determining vitamin A status for all five states of nutriture (Sommer et al., 1983; Sommer, 1994).

Vitamin A deficiency is most prevalent in developing countries (WHO, 1995) where staple crops make up the majority of the diet. Generally, the staple crops contain a small concentration of carotenoids; however, rice is the main exception and contains no carotenoids (McLaren and Frigg, 2001). Communities that are rice-dependent are more vulnerable to vitamin A deficiency. This is complicated by the fact that vitamin A is important for the resistance to infectious disease and the incidence of disease is often high in developing countries (Beaton et al., 1994 and Sommer and West, 1996). Vitamin A supplementation has been shown to reduce the risk of mortality associated with

these diseases in developing countries (Humphrey et al., 1996; West et al., 1999). Controlling infections, providing food fortification, conducting dietary interventions, and introducing genetically modified plants have been and continue to be the focus for controlling vitamin a deficiency. Children under the age of six and pregnant and lactating women constitute the main vulnerable groups in communities where vitamin A deficiency has been identified as a public health problem; supplementation should be considered for these individuals wherever it is deemed appropriate (McLaren and Frigg, 2001).

1.4 Models for studying vitamin A function in embryonic development

The importance of vitamin A throughout the life cycle has been well established (Moore, 1957; Wolf, 1984). As early as the 1930s, maternal insufficiency of vitamin A during pregnancy was recognized to result in incomplete pregnancies, fetal death, and severe congenital malformations (Zile, 1999). These congenital abnormalities have been identified in various species; however, in humans the effects of vitamin A deficiency are often disguised by general malnutrition (Gerster, 1997). Multi-nutrient deficiency is a confounding factor, making it difficult to isolate vitamin A deficiency as the sole cause of an abnormality. Even though a direct link has not been made between vitamin A deficiency and congenital malformations in humans, circumstantial evidence is available, i.e. premature neonates with low vitamin A stores are at high risk of respiratory abnormalities (Shenai et al., 1995), to suggest that abnormalities

experienced in humans share some similarity with those described in other animals.

Similarly, excess retinoids can have a deleterious effect on embryonic development. The malformations observed due to either vitamin A deficiency or excess appear to overlap; however, there are examples of selective responses to only deficiency or excess (Ross et al., 2000). Due to the strong evidence that human embryonic exposure to excess vitamin A results in congenital abnormalities (Kochhar et al., 1993; Rosa, 1993), treatment with retinoids is not permitted during pregnancy (Chan et al., 1996).

The requirement for vitamin A begins early in embryonic development (Zile, 2001). As evidenced by the severe malformations associated with deficiency and excess retinoids, developing embryos and fetuses require a precisely regulated supply of vitamin A. Recently, cell and molecular biology research, along with developmental studies have provided a wealth of information on the function of vitamin A in development. However, it was the discovery of the nuclear receptors for retinoic acid (RARs) and the retinoid X-receptors (RXRs) that revealed how RA can exert an effect at the gene level (Mangelsdorf et al., 1995; Pfahl and Chytil, 1996).

1.4.1 Mammalian models

Advances in molecular developmental biology, as well as the discovery of retinoid nuclear receptors, the RARs and the RXRs, have provided researchers with new methods to study the genomic role of vitamin A in mechanisms in

development. RA via activating the RAR-RXR hetrodimers, which then binds to the RARE in promoter regions of target genes, controls diverse, yet interconnected biological processes (Ross et al., 2000). Transgenic mice with alterations in retinoid receptor gene structure have been extensively used to examine the role of vitamin A in development, focusing on the specific function of different receptors during embryogenesis (Chambon, 1993; Kastner et al., 1994; Sucov et al., 1994; Boylan et al., 1995; Giguere et al., 1996). Although retinoid receptor isoforms have unique distribution patterns during embryonic development (Durston et al., 1997), single isoform knockouts have failed to produce phenotypic abnormalities (Kastner et al., 1995), due to retinoid receptor redundancy (Luo et al., 1995). Developmental malformations can arise as a result of knocking out the entire gene for a retinoid receptor; however, embryogenesis is well protected by a system that allows most or all functions of an absent receptor to be substituted by another (Ross et al., 2000). Receptor knockouts have provided valuable information into the function of vitamin A in development; however, they alone cannot provide definitive answers.

Vitamin A-deficient (VAD) animals can be used to study the molecular mechanisms of retinoid action by regulating the presence of retinoids and examining gene expression. Rats can be used to target retinoid deficiency to distinct gestational windows (White and Clagett-Dame, 1996; Wellik et al., 1997; Smith et al., 1998). Rat embryos made deficient in retinoids during gestational days 11.5-13.5 exhibit specific cardiac, limb, ocular, and nervous system deficits, some of which are partially similar to those reported in retinoid

receptor knockout mice, while others are novel (Smith et al., 1998). The retinoid deficient rodent model is useful; however, it is limited to studying later gestational stages, and there is some uncertainty about the exact vitamin A status of the embryo, since the embryo depends on the maternal supply for all nutrients, and the mother must have vitamin A for her own survival (Zile, 1999).

1.4.2 Avian embryo "retinoid ligand knockout" model

In contrast to the rodent model, the avian embryo model can be used to study vitamin A-dependent developmental events early in embryonic development (Zile, 1998; Zile, 2001). The initial discovery by Thompson et al. (1969) and subsequent work by Heine et al. (1985) has established that vitamin A is necessary for the establishment of the early avian vasculature. Importantly, RA fed to adult quail is not transported to the egg and thus completely VAD embryos can be obtained from RA sufficient adult quails (Dong and Zile, 1995; Chen et al., 1996). This avian embryo retinoid ligand knockout model provides an ideal system to study the physiological role of vitamin A in early development. Work with this model has already made several contributions to the understanding of the function of vitamin A in cardiovascular, hindbrain, somite, and limb development (Zile, 1999; Zile, 2001).

Vitamin A is essential for early embryonic development and VAD embryos die at day 3.5 of embryonic life (Dersch and Zile, 1993). Abnormalities in the cardiovascular system, head, central nervous system, hematopoietic organs, and the trunk are observed in VAD avian embryos (Dersch and Zile,

1993; Twal et al., 1995; Maden et al., 1996, 1998; Zile, 1998; Zile, 2001). Administration of vitamin A active compounds during the early stages of development "rescues" the VAD embryo and results in normal development (Dersch and Zile, 1993). Zile's group has discovered a retinoid-requiring developmental window, a stage in embryonic development concurrent with initiation of morphogenesis and the early stages in the formation of the cardiovascular system, during which there is an absolute requirement for vitamin A (Zile, 1998; Zile, 2001).

The VAD quail model in our laboratory has been used mainly to study the function of vitamin A in early cardiovascular development. These studies are of particular importance because of the obvious relevance to human cardiovascular malformations, some of which may be diet-related during pregnancy (Ross et al., 2000). The high incidence of vitamin A deficiency in developing countries may account for the increased incidence of heart malformations in these populations (Sommer et al., 1986). Very little is known about the etiology of congenital heart malformations. Further research is needed to describe the etiological factors, especially dietary ones during pregnancy, since diet directly contributes to the physiology and pathology of the body.

1.5 Avian cardiovascular development

Cardiovascular development is a complicated process and involves the simultaneous and interrelated development of the heart, the extra- and extraembryonic vascular networks and the subsequent linking of the heart and

the circulation. Cardiovascular development is conserved across species, especially in vertebrates, which have closely related developmental paradigms (Fishman and Chien, 1997). Many cells and tissues are involved in cardiovascular development; changes in cellular function and morphology are essential for proper development. Several signaling pathways have been identified to be involved in cardiovascular development; however, the whole pathway remains to be elucidated. Development of the cardiovascular system begins at an early developmental stage, with the simultaneous formation of the cardiogenic precursors and the extra- and intraembryonic blood vessels. Two basic types of processes are involved in cardiovascular development: those processes that drive morphological development, such as migration, proliferation, and apoptosis; and those processes that are necessary for functional development, including specification, differentiation, and determination of cardiogenic tissues (Farrell and Kirby, 2001).

1.5.1 Avian cardiogenesis

The heart is the first organ formed in the developing embryo and it serves as the physiological center of the cardiovascular system. On the other end of the life-spectrum, the cessation of a beating heart is often used as the definition of embryonic death. Proper regulation of cardiogenesis is required for embryonic development. Congenital heart malformations associated with developmental abnormalities during cardiogenesis account for 20% of all spontaneous human abortions (Hoffman, 1995). Cardiovascular development is fundamental to

vertebrate embryonic development and a large amount of work has been done to elucidate the molecular and developmental mechanisms of heart development.

The term heart-forming region (HFR) is used to describe the area in which cardiac progenitor cells arise. Figure 1 is a diagram of the relative positions of cells that will form the heart, following their migrations from the primitive streak, to the lateral plate, and finally to the heart tube during embryonic development (Fishman and Chien, 1997; Redkar et al., 2001). Cells in the HFR contribute to all layers of the mature heart (Redkar et al., 2001).

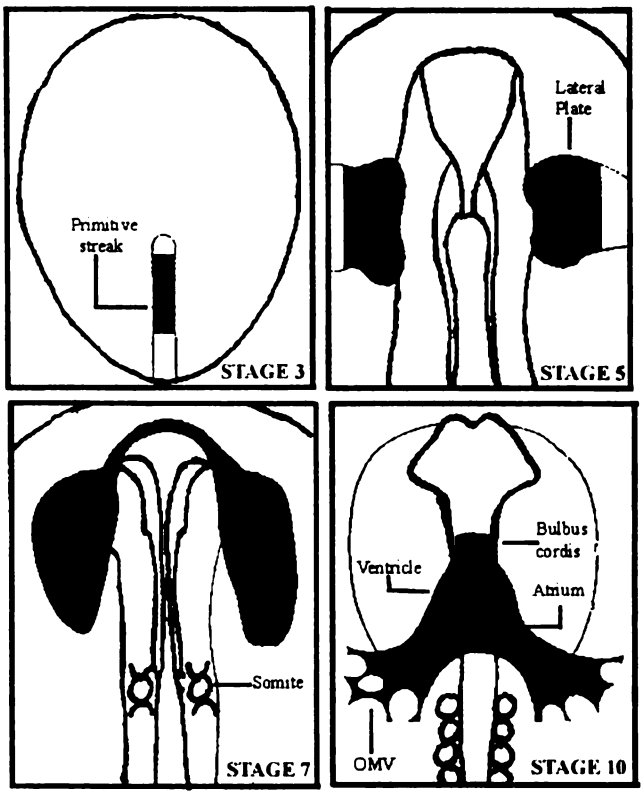

Figure 1. Schematic of the location of cardiogenic progenitors in the avian embryo. The relative anterior-to-posterior positions of precardiac cells in the primitive streak (HH stage 3) are retained in the lateral plate (as shown in HH stage 5 and 7), and in the heart tube at HH stage 10 (Fishman and Chien, 1997). Cardiogenic progenitors are shown in black. All views are ventral.

Cardiac progenitors arise from the mesoderm; however, mesoderm alone has been shown to be insufficient to generate heart tissue in explant cultures from amphibians (Lough and Sugi, 2000). Epimyocardial and endocardial cells are the first cardiogenic lineages that are specified. They arise from the induction of mesoderm via growth factors produced by the anterior endoderm (Lough and Sugi, 2000; Sugi et al., 2001). Figure 2 shows cross-sections of the HFR, depicting the cell layers that contribute to heart formation. The heart

begins as a paired tubular structure; however, the closure of the foregut draws the cardiac precursors toward the midline where the endodermal components fuse. Precursors of the endocardium cluster on the foregut side and are distinguished from premyocardial cells by the expression of QH-1 (Linask, 1992; Linask and Lash, 1993) and flk, and they lack N-cadherin expression (Linask, 1992) and the muscle genes. By HH stage 9-10, the two-endocardial tubes have fused to form one heart tube (Heine et al., 1985), which begins beating simultaneously (Fishman and Chien, 1997). See Table 1 for comparative chronology of heart development.

Table 1. Comparative Chronology of Cardiovascular Development[1]

	QUAIL	MOUSE	HUMAN
Primitive streak	HH 3	5-7 d	15-16 d
Assembly of lateral plate	HH 5	7 d	18 d
Heart-tubes fuse	HH 9	8 d	22 d
Heart starts beating	HH 10	8.5 d	23 d
Looping	HH 11	8.5 d	23 d
Birth	HH 46 (17-18 d)	19.5 d	40 wk

1 Data from: Sissman, 1970 and Fishman and Chien, 1997

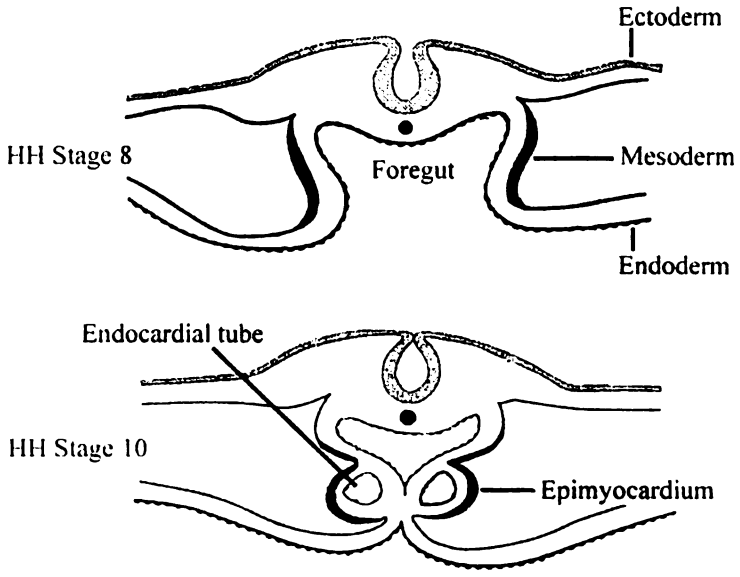

Figure 2. Cross-sections through the avian embryonic HFR. Cardiogenic progenitors in the mesoderm respond to inductive signals from the endoderm. By HH stage 9-10 the endocardial tubes fuse, forming one heart tube, surrounded by epimyocardial and epicardial cells. Adapted from the Atlas of Descriptive Embryology, 4th Edition.

Only the earliest developmental events in cardiogenesis have been reviewed here. Reviews by Olson and Srivastava (1996), and Kirby and Waldo (1990) describe in detail the later stages of cardiac morphogenesis. Briefly, after the heart-tubes fuse, the heart orients on the right side of the embryo. As the heart continues to develop and grow, the posterior and anterior ends remain in fixed positions and the heart loops to the right in all vertebrates; however, the exact mechanism for looping is not known. Recently, significant progress has been made defining the positional and molecular cues that guide heart looping and asymmetry (Levin, 1997; Beddington and Robertson, 1999; Brown and

Anderson, 1999; King and Brown, 1999; Majumder and Overbeek, 1999; Yost, 1999). It is clear that the asymmetry pathway is defined early in embryonic development and is tightly regulated with stimulatory and inhibitory signals.

Neural crest cells and head mesenchyme also contribute to heart development, specifically the formation of the outflow tract; these cells are located outside of the HFR. This points out the fact that cells outside the HFR need to be considered when examining heart development. Many factors contribute to heart development and although great strides have been made into the understanding of heart development, more research is still needed.

1.5.2 Avian vascular development

The vascular system arises early in embryogenesis to meet the nutritional needs of the developing organism (Risau, 1995). Two mechanisms are involved in embryonic vascular development: vasculogenesis and angiogenesis. Vasculogenesis is defined as the differentiation of mesodermal cells into endothelial precursors, which form the endothelial-lined blood vessels (Poole and Coffin, 1989, 1991; Risau, 1991); angiogenesis is the sprouting and branching of preexisting endothelial cells (Ausprunk and Folkman, 1977; Poole and Coffin, 1989; Noden, 1991). These two mechanisms are interconnected and together form the basis of vascular development.

The origin of endothelial cells and their assembly into the primary vascular system have been extensively studied in avian embryos (Pardanaud et al., 1987; Coffin and Poole, 1988; Poole and Coffin, 1989; 1991; DeRuiter et al.,

1993). The precursor cells of the extraembryonal vascular network arise from mesoderm in the area vasculosa. Precursor cells aggregate, forming blood islands starting at the head-process stage (Pardanaud, 1987). Wilt (1966) showed that differentiation of blood islands depends on an interaction between the mesoderm and the underlying endoderm. Thus, like in heart development, an inductive signal is needed for vascularization. In quail embryos this differentiation begins at the head-process stage and by the one-somite stage endothelial cells are detectable in the extraembryonic area (Coffin and Poole, 1988).

The blood islands consist of angioblasts, undifferentiated vascular cells that will become endothelial cells and blood cells, which form in the area vasculosa, and as the vascular system develops the blood island fuse to form the primary vascular plexus (Patan, 2000). Angioblasts respond to stimulatory and inhibitory signals, undergoing proliferation, differentiation, and apoptosis, shaping the extraembryonic vascular plexus.

Intraembyonal vasculogenesis occurs simultaneously with the development of the extraembryonal vascular plexus (Weinstein, 1999; Patan, 2000). Vascular development within the embryo is tightly correlated with heart development, and some cells arise from the same precursors in the mesoderm of the HFR (Inagaki et al., 1993). Endothelial cells can arise from the terminal differentiation of endothelial cell precursors within the HFR (vasculogenesis type I), or they can migrate to the HFR from the extraembryonal area pellucida (vasculogenesis type II) (DeRuiter et al., 1993). Endothelial cells are first

apparent around HH stage 6-7 in the chicken embryo (Linask, 1992). These cells assemble into a loose vascular plexus and coalesce into progressively larger tubes, until eventually generating a single endocardial heart tube, by HH stage 9 (Sugi and Markwald, 1996). Endothelial cells, the main component of the vascular system, line the heart as well as form the veins and arteries.

1.5.3 Avian inflow tract development

Although cardiogenic and vascular development are separate and distinct processes, they require interaction for the development of a fully functional cardiovascular system, evident at the inflow tract (IFT). A schematic diagram of the IFT at early heart morphogenesis is presented in Figure 3.

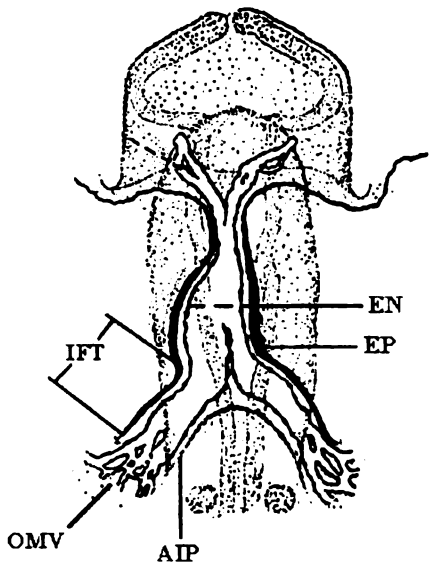

Figure 3. Schematic diagram of the avian IFT at HH10. The vascular endocardium (EN), surrounded by the epimyocardium (EP), connects to the extraembryonal vascular networks at the posterior region of the IFT through the omphalomesenteric veins (OMV). The EP forms the lateral boundary of the IFT and the anterior intestinal portal (AIP) forms the medial boundary. Adapted from Atlas of Descriptive Embryology, Fourth Edition, 1986.

Formation of the IFT requires cells from the HFR as well as vascular precursors from the extraembryonal area to amalgamate, forming a cohesive entity, consisting of an epimyocardial outer layer and an endocardial inner layer. The IFT serves as the transition point between the extraembryonic vascular plexus and the heart. As the intraembryonic vascular networks extend laterally, the extraembryonic vascular networks extend medially and the two systems fuse together by HH 9 at the IFT (Pardanaud et al., 1987; Coffin and Poole, 1988). In the avian embryo, blood arises from the blood islands located in the extraembryonal area vasculosa, and enters the atrium through the omphalomesenteric veins (OMV) at the inflow tract (Patan, 2000). IFT development is a complicated process, requiring proper mediation of cellular differentiation, proliferation, migration, adhesion, apoptosis, cell-cell recognition, cell-matrix interactions, and multiple cell lineages. Currently, there is insufficient literature describing IFT development.

1.5.4 Retinoids and cardiovascular development

As early as the 1930s it was recognized that maternal insufficiency of vitamin A during pregnancy results in fetal death or abnormalities in the offspring that include abnormal heart development (Mason, 1935). Initial clues into the function of retinoids in heart development came nearly 50 years ago, with studies of the effects of VAD diets on the pups of pregnant rats (Wilson et al., 1953). Thompson et al. (1969) made the important discovery that vitamin A is required for early avian embryonic cardiovascular development. Subsequent

studies by Heine et al. (1985) provided a descriptive analysis of the effects of vitamin A deficiency on avian cardiovascular development, in particular on the requirement of vitamin A for development of the early vasculature, with abnormalities in VAD embryos evident between the 5 and 15 somite stages. These observations were confirmed and extended using the quail embryo ligand knockout model (Dersch and Zile, 1993; Kostetskii et al., 1998; Zile, 2000). The discovery of a critical retinoid-requiring developmental window by Kostetskii et al. (1998) has made it possible to specifically address the role of vitamin A in early development. The abnormalities in heart development are the first pronounced morphological changes due to vitamin A deficiency in the early embryo (Zile, 1998, 2000, 2001); specifically, the earliest gross developmental change evident at the time of formation of the cardiovascular system in the VAD avian embryo is the abnormal IFT development (Zile, 2001). It is of interest to note that RA synthesis in the mouse heart has been localized to the IFT during early development (Moss et al., 1998), corresponding to the earliest morphological defects in cardiogenesis observed in the VAD quail embryo. Presently, it is unclear whether the abnormal IFT development in the VAD quail embryo is the consequence of abnormal heart morphogenesis, or if IFT development is a separate retinoid regulated process.

1.6 Retinoid-regulated molecular mechanisms of cardiovascular development

The discovery of nuclear receptors for RA and their hetrodimer partners, the RXRs has provided an explanation for the molecular mechanism by which retinoids control many physiological processes, including cardiovascular development (Mangelsdorf et al., 1995; Pfahl and Chytil, 1996). Vitamin A is involved in early heart and vascular development, most likely via the retinoid receptors. Our laboratory is studying the function of retinoids in cardiovascular development and has demonstrated that the early avian embryo expresses all the genes for retinoid receptors, and their expression includes the HFR (Kostetskii et al., 1998; Zile et al., 1997; Cui et al., in preparation). RARβ2 expression in the HFR is down regulated in the absence of vitamin A (Kostetskii et al., 1996, 1998). These findings in conjunction with the morphological studies on VAD quail embryonic cardiovascular development make it logical to think that retinoid receptors are involved in the transcriptional regulation of cardiovascular development. However, the interaction among many factors is required for cardiovascular development and the underlying mechanisms remain to be elucidated.

1.6.1 Cardiogenic genes regulated by retinoic acid

There are no master regulators that control heart development; therefore, the precise interaction among many genes is required for proper development (Laverriere et al., 1994; Evans, 1997; Fishman and Chien, 1997). Our laboratory

has examined the role of vitamin A in the regulation of various cardiogenic genes and has demonstrated that the heart specific gene, Nkx 2.5, as well as cardiac muscle specifying genes are not affected by vitamin A status (Kostetskii et al., 1999). It was discovered, however that the cardiogenic gene GATA-4 is altered in the VAD quail embryo (Kostetskii et al., 1999).

The GATA gene family encodes transcription factors involved in hematopoetic lineage determination and in cardiovascular development (Laverriere et al., 1994; Evans, 1997). GATA-4 expression has been reported to be retinoic acid responsive (Arceci et al., 1993), and our studies with the quail embryo retinoid-ligand knockout model have shown that GATA-4 is severely down regulated in VAD quail embryos, especially in the area of the posterior heart tube and subsequent inflow tract (Zile, 1998). It is possible that the abnormal morphology associated with vitamin A deficiency in the quail embryo may be due in part to downstream cardiac-specific genes that are regulated by GATA-4 via RA (Zile, 1999).

Vitamin A is also involved in determining cardiogenic asymmetry. In the retinoid ligand knockout quail embryo heart position is randomized (Zile et al., 2000). Heart sidedness can be rescued by administering vitamin A-active compounds by HH stage 8 (Kostetskii et al., 1998; Zile et al., 1998, Zile et al., 2000). These observations challenged the recently proposed molecular pathway for determining cardiac L-R asymmetry, because the asymmetric expression of all the early asymmetry genes including Shh in the quail Hensen's node, is not altered by vitamin A deficiency (Chen et al., 1996), and importantly, cardiac

asymmetry can be rescued by administering RA downstream of the Shh and other early asymmetry signaling pathways (Zile et al., 2000). It is clear that vitamin A is involved in the determination of cardiac asymmetry at the late stage of the asymmetry pathway; however, more research is needed to determine the molecular mechanisms of retinoid-regulated cardiac asymmetry aspects.

1.6.2 Vasculogenic genes and vitamin A status

Cell differentiation, proliferation, organization, and apoptosis are maintained under strict control to complete the development of a single, interconnected vascular system (Cleaver and Krieg, 1999). Recent work has identified molecules involved in early differentiation of mesodermal cells into endothelial cells, as well as molecules involved in endothelial cell proliferation, migration, and remodeling (Cleaver and Krieg, 1999). Growth factors are required for early differentiation of endothelial cell precursors, the angioblasts, located in the mesoderm, into endothelial cells. Members of the fibroblast growth factor (FGF) family play a critical role in the induction of the mesodermal germ layer during the earliest stages of embryogenesis (Cleaver and Krieg, 1999). Cell culture experiments have shown that FGF induces the expression of flk-1, the receptor for vascular endothelial growth factor (VEGF), which is required for angioblast differentiation (Eichmann et al., 1993; Cleaver and Krieg, 1999). Once angioblasts differentiate into endothelial cells they proliferate and migrate before assembling into blood vessels; both FGF and

VEGF are mitogens for endothelial cells (Keyt et al., 1996; Cleaver and Krieg, 1999).

The VAD embryo lacks a vascular link between the embryo and the extraembryonal vascular system (Thompson, 1969; Heine et al., 1985; Dersch and Zile, 1993; Twal et al., 1995; Zile, 1998; Zile, 1999; Zile, 2001). Thus, it is very likely that vitamin A is involved in the regulation of vasculogenesis at the gene level.

Extracellular matrix (ECM) can modulate growth and differentiation of endothelial cells and also influence their migration (Drake et al., 1992). These developmental events require the coordinated recognition and response of cell surface molecules to those in the ECM. Fibronectin (FN) is an ECM molecule that has been linked to endothelial cell motility during vascular development (Noden, 1991). Perturbations of normal avian embryogenesis with RA between HH stages 5 and 8 show similarities to cardiovascular defects arising in embryos treated with FN antibodies (Osmond et al., 1991) and in mouse embryos lacking FN (George et al., 1993). Furthermore, preliminary work by Linask and our laboratory has shown FN to be down regulated in VAD quail embryos (not published). Cells interact with the ECM via cell adhesion molecules. Integrins are the best characterized cell adhesion molecules and bind to collagen, laminin, fibronectin, and thrombospondin of the ECM (Cleaver and Krieg, 1999). When quail embryos are injected with CSAT, an antibody against β_1-integrin, vascular development is arrested at the stage when slender cord-like assemblies of angioblasts rearrange to form tubules (Drake et al., 1992). Some of the aspects

of retinoid-regulated vasculogenesis will be addressed in the present thesis research.

1.6.3 Transforming growth factor beta

The transforming growth factor betas (TGFβ) are a family of multifunctional polypeptide growth factors capable of mediating cell growth, differentiation, and morphogenesis (Koli et al., 2001) by modifying the expression of a specific set of target genes (Massague and Chen, 2000). One of the main functions of TGFβ is the inhibition of cell proliferation (Piek et al., 1999); however, the influence of TGFβ is dependent on the cell lineage, coexisting growth factors, and other biological and physiological variables (Brand and Schneider, 1995). The pleiotropic actions of TGFβ are best illustrated by their ability to exert either positive or negative effects on cell growth, illustrating the necessity to determine its biological role empirically in any given context (Brand and Schneider, 1995).

Members of the TGFβ family have been implicated in heart (Brand and Schneider, 1995; McCormick, 2001) and vascular development (Brown et al., 1999; Goumans et al., 1999; Dickson, et al., 1995). TGFβs have been shown to induce the generation of myocardium (Muslin and Williams, 1991; Slager et al., 1993) and to inhibit vascular development (Hayasaka et al., 1998; Zhao and Overbeek, 2001) in tissue explants; however, the role of TGFβ in early cardiovascular development has not been elucidated. It is known that the TGFβs are subject to regulation by retinoids (Mahmood et al., 1992; Salbert et al., 1993;

Mahmood et al., 1995; Han et al., 1997; Imai et al., 1997;Yoshizawa et al., 1998; Koli et al., 2001); members of the TGFβ family are candidate genes that mediate the downstream effects of RA in the early heart tube (Kubalak and Sucov, 1999).

TGFβ is typically secreted as part of a latent dimeric complex. The complex consists of a homo- or hetrodimer pro-peptide domain, TGFβ latency associated protein (LAP), which is noncovalently associated with the mature TGFβ dimer, and latent TGFβ binding protein (LTBP) covalently bound to LAP (Brand and Schneider, 1995; Lawrence, 1995; Koli et al., 2001). The associated proteins mediate proper folding and protect the mature peptide from degradation (Lawrence, 1995). Once secreted, the latent TGFβ complex associates with interstitial ECM and basement membranes (Roberts and Sporn, 1996). Mechanisms involving proteolysis are required to release and activate the pro-peptide domain (Koli et al., 2001). The active TGFβ binds to TGFβ serine/threonine protein kinase receptors, the TBRs, and elicits transcriptional activity via an intracellular signal transduction pathway that is depicted in Figure 4.

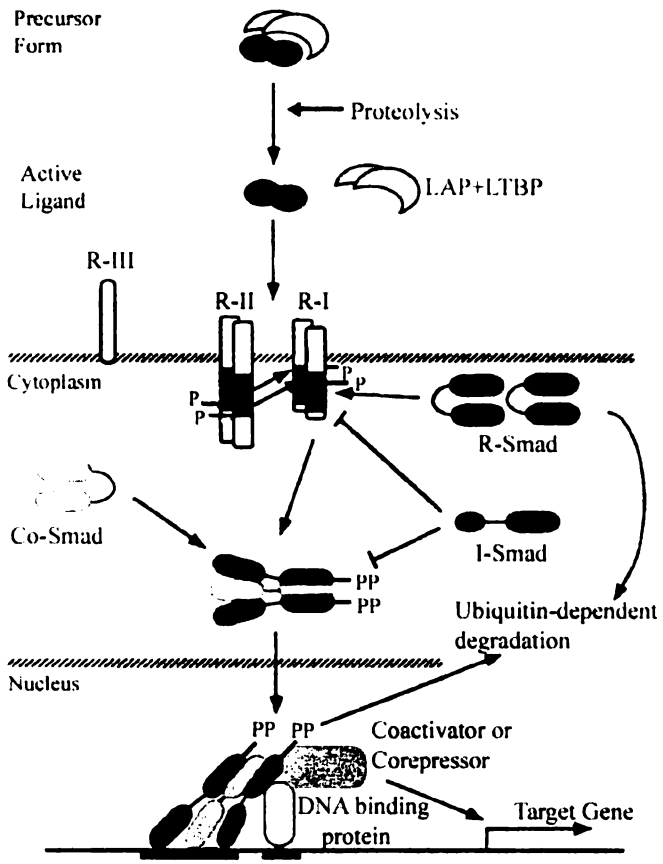

Figure 4. Signaling by TGFβ through its serine/threonine kinase receptors and Smad proteins. The active pro-peptide dimer, cleaved from LAP and LTBP by proteolysis, binds to a heterotetramer of the type II and type I TGFβ receptors R-II and R-I, respectively. Although not essential for signal transduction, the type III TGFβ receptor (R-II) can facilitate the binding of active ligands to R-II. I-Smads inhibit signaling by R-Smads:Co-Smad complexes by competitive inhibition. R-Smad:Co-Smad complexes bind to Smad responsive elements (SRE), DNA binding proteins, and coactivators to elicit transcriptional activity Adapted from Miyazono, 2000.

The regulation of TGFβ by retinoids is well recognized but is not understood. The promoters of the TGFβ genes do not contain RARE, indicating that an indirect method of regulation is involved (Roberts and Sporn, 1992a). Retinoids have been implicated in the activation of latent TGFβ post-

transcriptionally (Kojima and Rifkin, 1993; Yoshizawa et al., 1998); however, the mechanism remains unclear. Using both RA excess and VAD mouse embryos, Mahmood et al. (1992, 1995) have shown that retinoids are capable of differentially regulating TGFβ isoforms through mechanisms involving different stages in the process of TGFβ synthesis and secretion. Most of the current literature has focused on the increase in TGFβ expression associated with the addition of excess RA, either in cell culture (Kojima and Rifkin, 1993; Imai et al., 1997; Yoshizawa et al., 1998), or in mouse embryonic development (Mahmood et al., 1995). However, a recent study has shown that TGFβ2 is increased in RXRα knockout mice embryos, leading to increased apoptosis in the outflow tract of the heart (Kubalak et al., 2002). Similarly, RARα and RXRα proteins have been shown to inhibit TGFβ promoters via direct protein-protein interactions in a concentration-dependent manner (Salbert et al., 1993). Our findings that RARα2 and RARβ2 are severely down-regulated in VAD quail embryos (Cui et al., in preparation), and our preliminary findings of altered TGFβ expression in these embryos (Kostetskii et al., 1993), suggest a role of retinoids in the TGFβ signaling pathway during quail embryonic development, this forms the basis for the research described here.

CHAPTER 2

RATIONALE AND OBJECTIVES

The absolute requirement for vitamin A during embryonic development is well established. Studies with the VAD avian embryo model in our laboratory have provided important information regarding the cardiovascular phenotype attributed solely to the absence of RA signaling, i.e. an abnormal, non-looped heart with random left/right orientation, a thin-walled dilated heart cavity, defects in the outflow tract, and an absence of the inflow tract (IFT) that links the extraembryonal vasculature to the heart. Although these physiological defects of the cardiovascular system have been attributed to VAD, the molecular mechanisms remain to be elucidated. Our laboratory is focused on research concerning the function of vitamin A in cardiovascular development and has identified several retinoid-regulated genes that are linked to heart morphogenesis (Kostetskii et al., 1999; Zile et al., 2000; Zile, 2001). The lack of formation of the IFT as well as the identification of the down regulation of the GATA-4 gene in the posterior heart regions of the VAD embryo has led to hypothesize that the initial effect of vitamin A on heart development is at the site of cardiac IFT formation in the posterior HFR, as this is the earliest event in cardiac morphogenesis and the segmentation of the linear heart tube proceeds in a posterior to anterior direction.

Development of the IFT is a complicated process requiring the mediation of cell proliferation, differentiation, apoptosis, and migration and organization of

presumptive endothelial cells (angioblasts) from the extraembryonal area and mesodermal cardiogenic cell in the HFR to give rise to the IFT. The IFT are located bilaterally of the midline in the posterior HFR and contain the OMV that link the embryo with the extraembryonal vasculature. Thus, it is clear that many signaling pathways are involved in IFT development, and a likely candidate for RA regulation for these events would be a growth factor that has multiple regulatory functions. The TGFβ family of multifunctional polypeptides are possible candidate genes.

The TGFβ family of cytokines that regulate cell proliferation, differentiation, recognition, death, tissue recycling and repair (Massague, 1996), have been linked with cardiovascular development. The primary receptor for TGFβ, transforming growth factor-β receptor type II (TBRII) is present in endothelial cells and marks the extraembryonal vascular networks in quail and chicken (Brown et al., 1999). Also, TGFβ signaling is required for the organization of extraembryonic endothelial cells into robust vessels (Dickson et al., 1995; Goumans et al., 1999). The role of TGFβ in cardiovascular development is not limited to endothelial cells, as myocardial cells also express TGFβ during cardiogenesis (McCormick, 2001; Brand and Schneider, 1995; Roberts and Sporn, 1992a), and TGFβ2 knockout mice have congenital heart defects, mainly in the myocardium of the outflow tract (Sanford et al., 1997). It is well known that in many cells vitamin A regulates TGFβ signaling (Mahmood et al., 1992; Mahmood et al., 1995; Roberts and Sporn, 1992a). *In vitro* studies using bovine endothelial cells have shown that RA up-regulates the expression of

TGFβ receptors types I and II, resulting in enhancement of TGFβ activity (Yoshizawa et al., 1998). While our studies were in progress, work in mice has shown that knocking out the retinoic X receptor α results in elevated TGFβ2, which can contribute to abnormal outflow tract morphogenesis by enhancing apoptosis in the endocardial cushions (Kubalak et al., 2002). However, the regulation of TGFβ by vitamin A has not been studied in the development of the IFT. Furthermore, preliminary work in our laboratory demonstrated increased TGFβ mRNA in the early VAD quail embryo (Kostetskii and Zile, 1993). This suggested a role of TGFβ2 in embryonic development, possibly in cardiovascular morphogenesis and IFT formation.

Presently, little is known about the development of the IFT and in particular about the role vitamin A plays in its development. Previous work in our laboratory has identified a critical retinoid-dependent developmental window, the 4/5 somite stage of neurulation, at which time retinoids are absolutely required for development to proceed normally and for the embryo to survive (Kostetskii et al., 1999; Zile, 2001). The retinoid-dependent developmental window is concurrent with the beginning of heart morphogenesis and of the formation of the IFT. If biologically active retinoids are administered before, or during the retinoid-dependent developmental window to VAD embryos, then IFT development, as well as all other characteristics of the VAD phenotype are rescued and the embryo survives (Kostetskii et al., 1999).

The overall goal of my research is to contribute to the understanding of the function of vitamin A in cardiovascular morphogenesis, in particular

understanding the molecular function of vitamin A in IFT development focused within the retinoid-regulated developmental window. The specific goals of my research are as follows:

1) Compare normal and VAD cardiovascular development.

   There has not been a systematic description of cardiovascular abnormalities associated with VAD during early embryogenesis. It is imperative to determine the developmental stages when morphological changes associated with vitamin A deficiency occur for subsequent studies to address the molecular events that might be involved in these developmental processes, especially in the formation of the IFT. Normal and VAD cardiovascular development will be studied using light microscopy to examine gross morphological development; immunolocalization studies will be conducted to compare vascular development using QH-1 antibody as a biomarker for presumptive endothelial cells.

2) Compare TGFβ2 protein and mRNA and TBRII mRNA in normal and VAD quail embryos during early embryogenesis.

   Previously, we showed that TGFβ2 expression is increased in the VAD quail; however, spatio-temporal localization has not been examined in early avian embryogenesis. We will examine normal and VAD TGFβ2 protein and mRNA levels and localization during early embryogenesis.

If TGFβ2 is involved in IFT development, we would expect it to be expressed in the HFR. The expression of TBRII, the principal receptor for TGFβ2, will also be studied during early development to determine if its expression is altered in VAD embryo, and if the receptor expression corresponds with ligand expression.

3) Examine the specific role of TGFβ2 in cardiovascular IFT morphogenesis.

If TGFβ2 mRNA is increased in the HFR of the VAD quail embryo, we will then elucidate the role of TGFβ2 in development linked to the VAD phenotype. The increased TGFβ2 expression associated with vitamin A deficiency will be blocked using antisense oligonucleotides to TGFβ2. Morphology, vascular development (QH-1), TGFβ2 mRNA, and TBRII mRNA will be examined in VAD embryos injected with antisense oligonucleotide to TGFβ2.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals and diets

Quail (Coturnix coturnix japonica) were housed at the Michigan State University Poultry Research and Teaching Farm. Normal eggs were obtained from hens fed game bird chow (Purina Mills Inc., St. Louis, MO). Vitamin A-deficient (VAD) eggs were obtained from hens fed a semi-purified diet adequate in all nutrients, but with 10 mg of all-trans-retinoic acid added per kg of diet as the only source of vitamin A, as previously described by Kostetskii et al. (1996). All birds were hatched from normal eggs obtained from the Michigan State University Poultry Research and Teaching Farm. Birds that were designated to be in the normal control group were fed Purina starter/grower diet from birth until they began to lay eggs at 6-7 weeks of age at which time the diet was changed to Purina breeder diet. Birds that were designated for the VAD experimental group were fed a semi-purified starter diet, described in Table 2, and switched to a semi-purified breeder diet at 6-7 weeks, when the birds began to lay eggs. The eggs from the young birds were confirmed to be depleted of vitamin A by collecting the VAD eggs from the young birds daily and examining embryo morphology after three days of incubation. When all of the embryos from eggs from the young birds were VAD according to our previously described phenotype, the eggs were used in experiments. The birds were kept in cages with

a ratio of 2:1, females to males. The birds had continuous access to food and water, which they received fresh daily.

Table 2. Composition of the Semi-Purified Diets

INGREDIENT (g/kg)	STARTER	BREEDER
Soybean meal (47.5% Protein)	505.00	464.00
Dextrose (monohydrate)	383.41	380.33
Mineral mix[1]	44.86	44.86
Soybean oil	40.00	40.00
Cellulose	10.00	0.00
Calcium phosphate (dibasic)	4.50	13.90
Choline dihydrogen citrate	4.20	3.20
DL-methionine	3.50	3.50
L-lysine hydrochloride	2.50	1.00
Calcium carbonate	1.50	48.70
all-trans-retinoic acid	0.01	0.01
Biotin	0.0006	0.0006
Vitamin B12	0.03	0.03
Vitamin D3	0.03	0.03
Folic acid	0.005	0.005
Menadione sodium bisulfite complex	0.0168	0.0168
Nicotinic acid	0.08	0.08
Calcium pantothenate	0.03	0.03
Pyridoxine hydrochloride	0.015	0.015
Riboflavin	0.015	0.015
Thiamin hydrochloride	0.025	0.025
DL-alpha-tocopheryl acetate	0.10	0.10
Butylated hydroxytoluene	0.10	0.10

[1] Custom Mineral Mix, DYET #290013 (Dyets Inc., Bethlehem, PA)

3.2 The quail embryo retinoid ligand knockout model

Quail eggs were collected daily, and the eggs from normal and VAD groups were kept separately. The eggs were stored at 13°C, and used within one week. Eggs were incubated at 38.5°C, with 98% humidity and with a 2-hr rotation-cycle (Kostetskii et al., 1999; Zile et al., 2000). Embryos obtained from eggs from hens fed normal chow diet were termed "normal". Embryos from eggs of hens fed the purified VAD diet were termed vitamin A deficient, because the retinoic acid from the diet of hens cannot be transferred to the egg (Dersch and Zile, 1993; Dong and Zile, 1995).

3.3 Dissection

After incubation embryos were dissected from the yolk using microsurgical scissors and forceps (Fine Science Tools Inc., Foster City, CA), in a weigh boat containing phosphate-buffered saline (PBS) at RT (Kostetskii et al., 1999; Zile et al., 2000). Embryos were then placed in a petri dish containing PBS at RT under a Nikon SMZ-2T (Nikon Corporation, Japan) microscope, and the embryonal membrane was removed using forceps. Embryos were staged according to Hamburger and Hamilton (1951). Pictures for morphological studies were taken of the embryos in PBS using a Nikon 6006 camera attached to the microscope. Images in this thesis are presented in color. The embryos were then fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight.

3.4 Sectioning

To examine morphology at the cellular level, fixed embryos were stained with a Toluidine Blue solution (Kostetskii et al., 1999) and cross-sectioned. The methods used to embed the embryos in paraffin and to section the embryo have been previously described (Kostetskii et al., 1996). Embryos were washed with PBS twice for 10 min, and then dehydrated in an ascending EtOH series, 25%, 50%, 75%, and 100% for 15 min each. Embryos were stained with a Toluidine Blue solution overnight at RT, and then washed 3 times with EtOH, 10 min each. Embryos were then treated with isopropanol for 10 min at RT and then moved to a 1:1 mixture of xylene and paraffin at 65-70°C for an additional 10 min, followed by treatment with paraffin for 10 min at 65-70°C. Embryos were embedded vertically in paraffin blocks and allowed to harden overnight at 4°C. Paraffin blocks were removed from their molds the next day, and the embryos sectioned using a Spencer 820 Microtome (American Optical) at 10 μm using standard methods (Kaufman, 1992). The sections were collected in a continuous strand and placed in a water bath at 40°C. Sections were then placed on gelatin-coated slides, examined under a light microscope (Ernst Leitz Wetzlar, Germany), and the slides with desired sections were incubated in a 37°C oven overnight to remove all moisture. Next, slides were dipped in xylene to remove paraffin, then dipped in MeOH and placed in a 37°C oven for ten minutes to dry. The sections were examined using a Nikon Optophot-2 compound microscope that was equipped with a Nikon 6006 camera and pictures were taken of representative sections.

3.5 *In ovo* microinjection

Exogenous compounds were administered to the developing embryo *in ovo* via microinjection, as described previously (Kostetskii et al., 1999; Kostetskii et al., 1998). Eggs were incubated for 22-28 hr at 38.5°C at 98% humidity with the eggs placed horizontally. The technique described by Selleck (1996) was used for windowing of eggs and for *in ovo* injection. The eggs were removed from the incubator and washed with 70% EtOH. A one ml syringe was used to remove 0.8 ml of egg albumin from the egg and the needle hole was sealed with masking tape. A piece of masking tape was placed on the side of the egg that was facing up during incubation and cut to lay flat. The tape stabilized the eggshell, and a window was cut inside the edges of the tape, leaving one side of the window intact to act as a hinge. Air bubbles were removed from the egg with a 1 ml syringe. Embryos were sub-blastodermally microinjected, using a 10 µl Hamilton syringe (Hamilton Company, Reno, NV). The embryos visible above the ink background were staged according to Hamburger and Hamilton (1951). The eggshells were sealed with masking tape. Sealed eggs were incubated horizontally for an additional 10 hr to 2 days in an incubator at 38.5°C with 98% humidity.

3.5.1 Rescue of the VAD embryos with vitamin A-active compounds

Kostetskii et al. (1998) were able to rescue VAD embryos by injecting biologically active retinoids. Previously, our lab discovered a critical retinoid-dependent developmental window at the 4-5 somite stage of development (mid-

neurulation) (Zile, 2001). The presence of vitamin A-active compound at this stage is essential for further development to take place normally. The VAD embryo can be rescued if vitamin A-active compounds are provided at or prior to this developmental window. In the present work, VAD embryos were treated during this developmental window so as to determine if their development could be rescued.

Yellow lighting in the room was used when handling retinoid compounds. Retinoids were kept under nitrogen and dissolved in EtOH. Approximately 3 mg of either ROH or RA was weighed out and dissolved in 1 ml of EtOH. The retinoid solution was diluted 1:1000 in EtOH and the concentration and spectra of retinoids in the solution was determined using a CARY 3E spectrophotometer (Varian, Inc., Palo Alto, CA). Absorbance and spectra were recorded from 400-200 nm. A second peak, or an alteration in the main peak characteristic for the retinoid signified degradation of the retinoid compounds, in which case the retinoids were discarded. All-trans-ROH has a major peak at 326 nm and a minor peak at 230 nm; all-trans-RA has a broad absorption with a maximum at 350 nm. Using a Hamilton syringe embryos were sub-blastodermally injected with 10μl of Tyrode's solution containing 10% VAD egg homogenate, 5% filter-sterilized Pelikan Fount India ink, and 10 ng of biologically active retinoids, either all-trans-ROH or all-trans-RA (Sigma Aldrich, St. Louis, MO); control solution contained the vehicle alone. Embryos at 3-5 somite stages were selected for experiments. The eggs were then sealed, and incubation was continued for an

additional 10 hr to 2 days. Normal and VAD control embryos were injected with the vehicle.

3.5.2 Blocking of development with anti-all-trans-retinoic acid monoclonal antibody

RA is the transcriptionally active form of vitamin A. Twal et al. (1995) showed that a monoclonal antibody against all-trans-RA blocked normal quail development when injected under the blastoderm before the retinoid-dependent developmental window. In the present study, anti-all-trans-RA MoAb was used to block vitamin A mediated development by sequestering all-trans-RA. The antibody producing cells were grown in culture in our lab and the MoAb secreted by the cells into mediums was purified using an affinity column (Twal et al., 1995). Antibody concentration was determined using a Spectronic 21D spectrophotometer (Spectronic Instruments, Rochester, NY).

Normal and VAD embryos were sub-blastodermally injected, using a 10μl Hamilton syringe, with 10 μl of Tyrode's solution containing 10% VAD egg homogenate, 5% filter-sterilized Pelikan Fount India ink, and 5 μg of anti-RA MoAb. Normal and VAD control embryos were injected with the vehicle alone, or mouse IgG (Sigma). Embryos at 1-3 somite stage were used for blocking studies. The eggs were sealed and incubated for an additional 10 hr to 2 days.

3.6 Immunohistochemical localization of endothelial cells

Monoclonal antibodies against QH-1, isolated from cells obtained from the Developmental Studies Hybridoma Bank at the University of Iowa and grown in our laboratory, were used to identify endothelial cells in quail embryos (Twal and Zile, 1997). Embryos were dissected from extraembryonal membranes and staged according to Hamburger and Hamilton (1951). Embryos were fixed overnight in 4% PFA in PBS and then rinsed 3 times in PBS, 30 min each. The embryos were then treated for 15 min with PBS containing 1% Triton X-100 (PBST) and next treated for an additional 15 min with hyaluronidase, 2 mg/ml in PBS. Blocking of nonspecific binding was performed by first incubating the embryos for 5 min with PBST containing 0.01 M glycine (PBST/G) and then for 1 hr with PBST/G containing 10% normal goat serum. Embryos were next incubated overnight at 4°C with, undiluted cell supernatant containing QH-1 antibody. Normal and VAD control embryos were incubated in Dulbecco's Modified Eeagle's Medium without the QH-1 antibody. Embryos were rinsed 3 times (1 hr each) with PBST and incubated for 2 hr with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Molecular Probes; Eugene, OR) at a 1:50 dilution in PBS. Prior to incubation, the goat IgG was preincubated for 1 hr with quail embryo acetone powder to reduce nonspecific binding. Embryos were then rinsed 2 times (30 min each) with PBS and once for 5 min with PBS containing 0.003% Evans blue. After 2 brief rinses with PBS the embryos were mounted in 40% PBS in glycerol and examined with an epifluorescence equipped Nikon Optiphot-2 compound microscope. Pictures

were taken with a Nikon 6006 camera mounted on the microscope. All steps were performed at RT except where indicated.

3.7 Immunohistochemical localization of TGFβ2

Rabbit polyclonal antibodies against TGFβ2 were generously provided by Dr. Anita Roberts at the National Cancer Institute (Bethesda, MD). Embryos were fixed in 4% PFA, then rinsed 3 times in PBS, 30 min each, and then 3 times with PBS containing 1% Triton-X (PBST), 10 min each. The embryos were then treated 2 times, 1 hr each, with PBST containing 2% instant skim milk (PBSMT) to solublize membranes and block nonspecific binding. The embryos were then incubated for 15 min with 2 mg/ml hyaluronidase in PBS to further block nonspecific binding. Embryos were next incubated overnight at 4°C with the TGFβ2 antibody diluted to a working concentration of 1 μl/ml in PBSMT. Embryos were rinsed 5 times with PBSMT, one hr each, and incubated overnight at 4°C with tetramethylrhodamine isothiocyanate (TRITC) –labeled donkey anti-rabbit IgG (Sigma Aldrich) at a 1:100 dilution in PBSMT. Prior to incubation, the donkey IgG was preincubated for 1 hr with quail embryo acetone powder. Embryos were rinsed 5 times, 1 hr each, with PBSMT, then for 20 min with PBST, and mounted in 50% PBS in glycerol, and examined with an epifluorescence equipped Nikon Optiphot-2 compound microscope. Pictures were taken with a Nikon 6006 camera mounted on the microscope. Normal and VAD control embryos were incubated in PBSMT with non-specific mouse IgG instead of the TGFβ2 antibody.

3.8 Analysis of TGFβ2 mRNA expression by *in situ* hybridization

3.8.1 Recovery of plasmid

cDNA for TGFβ2 (1.9 kb) was a gift from Dr. Anita Roberts at the National Cancer Institute. cDNA was inserted into the polyclonal region of puc19 vector. The obtained plasmid was on filter paper. To reconstitute the plasmid, the filter paper was placed in a sterile microfuge tube with 50 µl of sterile ddH_2O and vortexed 3 times for 5 min, and then stored at 4°C overnight. The filter paper was removed from the supernatant and the supernatant containing the plasmid was stored at -20°C. To verify the presence of the plasmid in the supernatant, 2 µl of the supernatant was mixed with 3 µl of DNA loading buffer, subjected to electrophoresis on 1% agarose gel, and stained with ethidium bromide.

3.8.2 Transfection of *E. coli* with plasmid containing TGFβ2 cDNA

Invitrogen One Shot TOP10 transformation kit (Invitrogen, Carlsbad, CA) was used to transfect *E. coli*, competent cells, with the plasmid containing the TGFβ2 cDNA. All components used in the reaction were obtained from the kit, except the supernatant containing the plasmid from above. The reaction was carried out on ice. The vial of competent cells was thawed on ice, then 2 µl of 0.5M β-mercaptoethanol was added and the cells allowed to stand on ice for 20 min, then 10 µl of the above supernatant containing the plasmid was added to the mixture. The competent cells were subjected to heat-shock in a 42°C water bath for 35 sec and then returned directly to the ice bath for 2 min. Regular LB

media, 250 µl, was added to the reaction mixture and incubated at 37°C in a water bath for 1 hr with vigorous shaking. The mixture was plated on LB plates containing ampicillin (LB/Amp) and incubated at 37°C overnight and then stored at 4°C until use. Colonies that grew contained the transfected plasmid.

3.8.3 Purification of plasmid

Single colonies that grew on the LB/Amp plate were inoculated in 3 ml LB/Amp media in sterile test tubes and incubated at 37°C in a water bath overnight with vigorous shaking. Promega Wizard Plus Minipreps DNA purification system (Promega, Madison, WI) was used to isolate the plasmid. All solutions were from the kit unless specified. The bacteria were centrifuged at 10,000 g for 10 min and the supernatant poured off. Resuspension solution, 300 µl, was added to the test tube, mixed by vortexing, and then transferred to a microfuge tube. Lysis solution, 300 µl, was added, the tube closed and mixed by inverting until the solution was clear, then 300 µl of neutralization solution was added and the sample centrifuged at 10,000 g for 5 min. An affinity microcolumn provided in the kit for the purpose of isolating the plasmid was placed on a 5 ml syringe and the plunger removed. Resin solution, 1 ml, was added to the barrel of the syringe, then the supernatant was added to the syringe and the solution was pushed through, trapping the plasmid in the microcolumn. The microcolumn was removed from the syringe, then the plunger, and then the microcolumn was placed back onto the syringe. Two ml of wash solution containing EtOH was placed in the syringe and pushed through precipitating the

plasmid. The microcolumn was removed from the syringe and placed in a sterile microfuge tube and subsequently centrifuged at 10,000 g for 2 min. The microcolumn was removed, and placed in a sterile microfuge tube, then 50 µl ddH_2O was added to the microcolumn and allowed to stand for 1 min, followed by centrifugation at 10,000 g for 25 sec. To verify the presence of the plasmid, 2 µl of the collected solution, hereafter referred to as purified plasmid, was mixed with 3 µl of DNA loading buffer, subjected to electrophoresis on 1% agarose gel, and stained with ethidium bromide. The purified plasmid was stored at -20°C.

3.8.4 Sub-cloning of TGFβ2 cDNA into pBSK$^-$

The cDNA for TGFβ2 was obtained in pUC19 vector, which does not contain an mRNA promoter. In order to make an mRNA riboprobe the cDNA was sub-cloned into a vector that contains an mRNA promoter, pBSK$^-$ (Stratagene, LaJolla, CA). Basic Local Alignment Search Tool (BLAST) was used to check for homology between TGFβ2 and other known genes in the NIH database. A unique sequence was chosen that had no known homology with any other avian genes. The pBSK$^-$ vector and the purified pUC19 plasmid containing the TGFβ2 cDNA were digested with Kpn1 and EcoRV (New England Biolabs, Beverly, MA) at 37°C for 3 hr. Digestion with Kpn1 and EcoRV cut a 40 bp section out of the polyclonal region of the pBSK$^-$ plasmid, and excised a 500 bp section of the cDNA unique to TGFβ2 from the pUC19 plasmid. Complete digestion was confirmed via electrophoresis on 1% agarose.

The entire sample from digestion was subjected to electrophoresis on 1% agarose at a 1:1 ratio with DNA loading buffer. QIAEX II Gel Extraction Kit (Qiagen, Inc., Valencia, CA) was used to isolate the digested pBSK$^-$ and the TGFβ2 segment. All solutions used for extraction were those provided in the kit, unless specified. The corresponding DNA bands were cut out from the agarose gel and placed in clear, sterilized microfuge tubes. The gel was weighed and 3 volumes of Buffer QX1 was added to every volume of gel containing the DNA fragments. QIEAX II was resuspended by vortexing and 10 µl of QIAEX II was added to the sample. The sample was incubated at 50°C for 10 min to solubilize the agarose and bind the DNA to QIEAX II. The sample was mixed by vortexing every 2 min during the incubation to keep the QIAEX II in suspension. The sample was centrifuged for 30 sec at 10,000g and the supernatant was discarded. The pellet was washed with 500 µl of Buffer QX1, resuspended by vortexing, centrifuged at 10,000g for 30 sec, and then the supernatant was discarded. The pellet was washed twice with 500 µl of Buffer PE as described above. After removing the supernatant from the second washing, the pellet was allowed to air-dry until it was white and dry. The DNA was eluted by adding 20 µl of sterilized ddH_20 to the pellet and incubating at RT for 5 min. The sample was centrifuged for 30 sec at 10,000g and the supernatant, which contains the DNA, was pipetted into a sterile microfuge tube. Elution was repeated a second time to recover any remaining DNA and to increase yield.

The isolated TGFβ2 DNA fragment was then ligated into the isolated pBSK$^-$ that had been digested, using T4 DNA ligase (Boehringer Mannheim,

Indianapolis, IN). pBSK$^-$, 0.1 μg, and an equimolar amount of the TGFβ2 DNA fragment were incubated in a sterile microfuge tube with 7.5 μl of sterile ddH_20, 0.1 Weiss unit of T4 DNA ligase, 1 μl 10X ligation buffer (supplied with the enzyme), and 1 μl of 5 mM ATP. Two control reactions were conducted: one using the pBSK$^-$ vector alone and another using only the TGFβ2 DNA fragments. The samples were incubated at 16°C overnight. The ligated samples were transfected into *E. coli* following the protocol described in Section 3.8.2, with modifications. LB/Amp plates, used to culture the bacteria, were treated with 40 μl of 20 mg/ml 5-Bromo-4-chloro-3-idolyl-β-D-galactopyranoside (X-gal)(Boehringer Mannheim) and 4 μl of 250 mg/ml Isopropyl-β-D-thiogalactoside (IPTG) (Boehringer Mannheim) for Blue/White screening. The recombinant colonies (white) were selected for further analysis. The plasmid was purified following the protocol outlined in Section 3.8.3.

3.8.5 Synthesis of TGFβ2 riboprobe

The purified plasmid was digested with Kpn I (New England Biolabs) for sense probe and Eco RV (New England Biolabs) for antisense probe synthesis as follows: 5 μl of purified plasmid, 3 μl of 10X buffer (supplied with the enzyme), 1 μl of the enzyme, 0.2 μl of bovine serum albumin (BSA) and 11.8 μl of diethyl pyrocarbonate (DEPC) treated ddH_20 was placed in a DEPC treated microfuge tube and incubated in a water bath at 37°C for 2.5 hr. To verify complete digestion of the plasmid, 2 μl of the digested plasmid was mixed with 3 μl of

DNA loading buffer, subjected to electrophoresis on 1% agarose gel, and stained with ethidium bromide. The digested plasmid was stored at -20°C until use.

In vitro transcription was carried out using Ambion T3 and T7 transcription kits (Ambion Inc., Austin, TX), for the preparation of sense and antisense probes, respectively. Two µl of digested plasmid, 2 µl of 10X transcription buffer, 2 µl ATP, 2 µl CTP, 2 µl GTP, 2 µl DigUTP:UTP (1 part digoxigenin-11-UTP (Roche Molecular Biochemicals, Indianapolis, IN):2 parts UTP), 6 µl DEPC treated H_20, 1 µl RNase inhibitor, and 1 µl of transcription enzyme was added to DEPC treated microfuge tubes and incubated in a water bath for 3 hr at 37°C. The probes were subjected to polyacrylamide gel electrophoresis (PAGE) stained with ethidium bromide to determine the size and concentration of the final products.

The samples were then treated with 1 µl of DNase (Ambion Inc.) at 37°C for 20 min and purified by EtOH precipitation as follows: 3 volumes of cold EtOH, 10 µg tRNA (Boehringer Mannheim), and $1/10^{th}$ volume of sodium acetate, 3M, pH 5.2 was added to the sample and stored at -20°C overnight. The sample was then centrifuged for 20 min at 4°C and 10,000g and the supernatant decanted. The sample was washed with 70% EtOH, dried in a Cenco Hyvac 14 speedvac (Central Scientific Company, Chicago, IL), and the pellet dissolved in 100 µl DEPC-H_20. The probes were subjected to PAGE and stained with ethidium bromide to determine the size and concentration of the final product.

3.8.6 Analysis of TGFβ2 expression by whole-mount *in situ* hybridization

Embryos were dissected from extraembryonal membranes and fixed in 4% PFA in PBS overnight at 4°C, then dehydrated through an ascending MeOH series (25%, 50%, 75%, 100%) and stored at -20°C. Embryos were rehydrated through a descending MeOH series (75%, 50%, 25%) at 30 min intervals and then washed 2 times with 1% polyoxyethylene sorbitan monolaurate (Tween 20) in PBS (PBT), 10 min each. The embryos were postfixed in 4% PFA/PBS for 30 min and then washed with PBS 3 times, 10 min each. Hybridization solution [50% Formamide (Roche Molecular Biochemicals; Indianapolis, IN), 5X SSC (20X: 3M NaCl, 0.3M sodium citrate, pH 7.0), 2X Denhardt's (50X: 0.5g Ficoll, 0.5g polyvinyl pyrrolidone, 0.5g BSA in 50 ml ddH_20), 1% sodium dodecyl sulfate (SDS), 100 μg/ml DNA, 100 μg/ml RNA, and 50 μg/ml heparin] was used to wash the embryos for 10 min, to prehybridize the embryos for 4 hr at 55°C, and to incubate the embryos with the specific riboprobe at a 1:1000 dilution overnight at 59°C. Embryos were washed 2 times at 60°C with 2X SSC, 1% SDS in PBT, 1 hr each. Then, 0.5X SSC, 1% SDS in PBT was used to wash the embryos first at 65°C and then at 70°C, 90 min each. TBST (137mM NaCl, 3mM KCl, 25mM TrisCl pH 7.5 and 0.1% Tween 20) containing 10% heat-inactivated goat serum and 4% dry nonfat milk was used to block non-specific binding by incubating for 3 hrs at RT. Embryos were then incubated with anti-digoxigenin alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) at a 1:2000 dilution in TBST containing 0.5 mg/ml levamisole overnight at 4°C. The antibody was preabsorbed with 1% embryo acetone powder. Embryos

were washed 8 times, 30 min each, with TBST containing 0.5 mg/ml levamisole at RT and then overnight at 4°C. Embryos were washed with TBST containing 0.5% levamisole 2 times for 10 min and then 2 times with NTMT (100mM NaCl, 50mM $MgCl_2$, 100mM TrisCl pH 9.5,0.1% Tween 20, and 0.5 mg/ml levamisole) for 10 min each. Embryos were incubated with 4.5µl 4-nitroblue tetrazolium chloride (Roche Molecular Biochemicals; Indianapolis, IN) and 2.3µl 5-bromo-4-chloro-3-indolyl-phosphate (Roche Molecular Biochemicals; Indianapolis, IN) per ml of NTMT at RT in a dark place until color had developed. The embryos were washed 3 times with PBT at RT, 30 min each, and then stored in 60% glycerol in PBS at 4°C. Pictures were taken of the embryos using a Nikon 6006 camera attached to a Nikon SMZ-2T light microscope. Experimental embryos were incubated with antisense probes, while control embryos were incubated with sense probes.

3.8.7 Cellular analysis of TGFβ2 expression by cross-sectional examination

The embryos used for whole-mount *in situ* hybridization were sectioned according to the protocol outlined in Section 3.4 with modifications. The embryos were not stained with the Toluidine Blue solution; instead they were placed directly in isopropanol after dehydration in EtOH. The embryos were embedded in paraffin, sectioned, and deparaffinized. The sections were examined using a Nikon Optophot-2 compound microscope. The microscope was equipped with a Nikon 6006 camera, and pictures were taken of representative sections.

3.9 Analysis of TBRII expression by *in situ* hybridization

3.9.1 Synthesis of TBRII riboprobes

cDNA for TBRII was a gracious gift from Dr. Barnett at Vanderbilt University. The cDNA was obtained on filter paper and the plasmid was recovered as described in Section 3.8.1. The plasmid was then transfected into *E. coli* as outlined in Section 3.8.2. The cDNA for TBRII was cloned into the xba1 site of pcDNA3. The vector, pcDNA3, contains mRNA promoters, so it was not necessary to sub-clone the cDNA into pBSK$^-$.

The protocol outlined in Section 3.8.5 was used to synthesize riboprobes, with alterations. First, BLAST was used to check for homology between TBRII and other known genes in the NIH database. A unique sequence was chosen that had no known homology with any other avian genes. The plasmid was digested with pvu I (New England Biolabs) for both sense- and antisense probe formation at the same concentrations specified previously. *In vitro* transcription was completed using Ambion T7 and Sp6 transcription kits for sense and antisense probe formation, respectively, as described in Section 3.8.5.

The sense probe was treated with 1 µl of DNase at 37°C for 20 min and then purified by EtOH precipitation. The resultant pellet was dissolved in 100 µl DEPC-H_20. The probe was subjected to PAGE stained with ethidium bromide to determine the size and concentration of the final product.

The anti-sense probe, ~1.8kb, was subjected to alkaline hydrolysis to reduce the average probe length (Cox et al., 1984). One µl of DNase and 1 µl of RNase were added to the anti-sense probe solution formed during *in vitro*

transcription. The sample was diluted to 100 µl using DEPC-H_2O. Hydrolysis buffer (80 mM sodium bicarbonate, 120 mM sodium carbonate, 20 mM β-mercaptoethanol), 100 µl, was added to the sample and incubated in a 60°C water bath for 30 min. The sample was removed from the water bath and 200 µl of Stop buffer (0.2 M sodium acetate pH 6.0, 1.0% glacial acetic acid, 10 mM dithiothreteitol) was added to the microfuge tube to terminate the reaction. The probe was purified by EtOH precipitation as previously described, except 1 µl of tRNA was added to the reaction. The resultant pellet was dissolved in 100 µl of DEPC-H_20. The probes were subjected to PAGE and stained with ethidium bromide to determine the final size and concentration.

3.9.2 Analysis of TBRII expression by whole-mount *in situ* hybridization

Experimental embryos were hybridized with antisense probes and control embryos were hybridized with sense probes, described above. The protocol outlined in Section 3.8.6 was followed for *in situ* hybridization. Pictures were taken of the embryos using a Nikon 6006 camera attached to a Nikon SMZ-2T light microscope.

3.9.3 Cellular analysis of TBRII expression by cross-sectional examination

Embryos used for whole-mount *in situ* hybridization were sectioned according to the protocol outlined in Section 3.8.7. The sections were examined using a Nikon Optophot-2 compound microscope. The microscope was equipped with a Nikon 6006 camera and pictures were taken of representative sections.

3.10 Antisense oligonucleotide blocking of TGFβ2

A unique sequence for TGFβ2 (5'-GCA CAG AAG TTG GCA TTG TAT CCT TTG GGT TC-3') previously described by Jakowlew et al. (1990) was chosen for the synthesis of antisense oligonucleotides. The oligonucleotides were synthesized at the Macromolecular Structure Facility at Michigan State University. The lypholized oligonucleotides were reconstituted in 100 μl of Tyrode's solution. The concentration of the oligonucleotides was determined in a Spectronic 21D spectrophotometer and the oligonucleotides were diluted to a working concentration of 50 μmol in Tyrode's solution. The oligonucleotide solution was sub-blastodermally injected into 1-2 somite stage embryos as described in Section 3.5. The eggs were sealed and incubated horizontally at 38.5°C with 98% humidity for 10 to 24 hr. Normal and VAD control embryos were injected with either the vehicle alone, or a non-specific oligonucleotide. The embryos were dissected as described in Section 3.3 and pictures were taken using a Nikon 6006 camera attached to the microscope. Embryos were then fixed overnight in 4% PFA and stained and sectioned as described in Section 3.4.

CHAPTER 4

RESULTS

4.1 VAD embryos display abnormal IFT development during early cardiac morphogenesis

The VAD avian embryo dies at 3.5 d of embryonic development (Kostetskii et al., 1999; Zile, 2001). The most pronounced developmental defect observed in the VAD avian embryo is the abnormal cardiovascular development (Thompson et al., 1969; Heine et al., 1985; Zile, 2001), which is likely the cause of the early embryo lethality observed in the VAD avian embryo, due to the lack of nutrients and growth factors that are normally provided to the developing embryo via the circulation. Our laboratory is focused on studying the effects of VAD on quail embryonic cardiovascular development and the molecular pathways that are responsible for the characteristic phenotype of the VAD quail embryo (Zile, 2001). Our lab has identified a critical retinoid-regulated developmental window at 4/5 somite stage (ss), at which time biologically active vitamin A is essential for normal embryonic development and survival (Kostetskii et al., 1999). Currently, there has not been a systematic description of cardiovascular abnormalities in the VAD embryo, in particular focusing on the inflow tract which connects the embryonic and extraembryonic vascular networks and is absent in the VAD avian embryo (Heine et al., 1985; Dersch and Zile, 1993; Zile, 1999; Zile et al., 2000; Zile, 2001). It is imperative to determine the developmental stages when morphological changes associated with

VAD occur, for subsequent studies to address the molecular events that might be involved in these developmental processes. Therefore, we examined morphological cardiovascular development of normal and VAD embryos starting at the critical retinoid-regulated developmental window (4/5 ss) through the time of VAD embryo lethality (3/3.5 d), focusing on IFT development.

No abnormalities were observed in VAD embryos between 4 and 7 ss. Cells in the HFR began to form a symmetrical, paired-tubular structure in both the normal and VAD embryos at 4/5 ss (Figure 5). The anterior intestinal portal (AIP) establishes the medial boundary of the HFR, as mesodermal cells differentiate into epimyocardium, they from the lateral boundary of the HFR. The fusion of the two heart-tubes at the mid-line (at 9 ss), determination of heart orientation and initiation of heart beating (at 10 ss), and heart looping (at 11/12 ss) were all affected in the VAD embryo (Figure 5). The fusion of the heart-tubes was delayed in the VAD embryo to 10/11 ss, and the single chambered, linear heart-tube became dilated. In the normal embryo the linear heart-tube orients on the right side of the midline; however, the determination of heart orientation was altered in the VAD embryo and heart orientation was randomized (heart shown on left in Figure 5). Shortly after heart-tube fusion (11/12 ss), the VAD heart began beating; however, the rate was slow compared to that of the normal heart. The IFT connects the embryo to the extraembryonal circulation and the nutrients stored in the yolk. In the VAD embryo, by 10 ss, a narrowing and a subsequent closure (by 11/12 ss) of the IFT was observed. The narrowing

of the IFT was the first observable morphological change in VAD cardiovascular development.

In the normal embryo by 2.5 d of embryonic development the extraembryonic vasculature forms a distinct network of veins and arteries for delivering nutrients from the yolk to the embryo via the omphalomesenteric veins (OMV) at the IFT and via the omphalomesenteric arteries (OMA) at the dorsal aorta to the posterior embryo (Figure 5). The contracting, two-chambered heart, oriented on the right side of the embryo, continuously pumps blood throughout the circulatory system. At this stage of development the entire VAD embryo had severe defects, including stunted growth and compressed somites. A single chambered heart with a dilated cavity and with randomized orientation was observed in the VAD embryo. Blood islands were present at the periphery of the area vasculosa: there was limited formation of blood vessel endothelium and there was no organization of cells into vascular networks. The IFT had closed, and there was a complete absence of OMV and OMA in the VAD embryo (Figure 5). The VAD heart continues to beat until 3-3.5 d, but there are no significant changes in embryo morphology during the last day of embryonic development.

Administration of atRA or all-trans-retinol before or during the retinoid-regulated developmental window (4/5 ss) completely rescued the VAD phenotype (Figure 5). Heart looping in the rescued embryos was normal and the heart was oriented asymmetrically on the right side of the embryo. Blood vessels coalesced into a well-defined vasculature, which connected to the embryo via the

OMV at the cardiac IFT and the OMA at the dorsal aorta. The rescued embryos were normal in size and in all other developmental aspects. Administration of biologically active retinoids to normal embryos at the concentrations used for rescuing VAD embryos, had no observable effects on development. To verify the role of atRA as the regulatory molecule in avian embryonic development, normal embryos were treated with anti-atRA MoAb. Microinjection of anti-atRA MoAb into normal embryos before the retinoid-regulated developmental window resulted in embryos displaying the complete VAD phenotype (Figure 5), including formation of a ballooned, single chamber heart, with randomized orientation, and an absence of vascular networks in the extraembryonal area. The OMV at the IFT and the OMA at the dorsal aorta were absent (Figure 5). Administration of anti-all-trans-RA MoAb to VAD embryos at similar stages had no further deleterious effects on development. Normal and VAD control embryos injected with vehicle had no effect on development. A small percentage of normal and VAD control embryos injected with mouse IgG had non-specific abnormalities, i.e. the heart was positioned above the head, or spontaneous death occurred; however, the majority of embryos had no developmental defects.

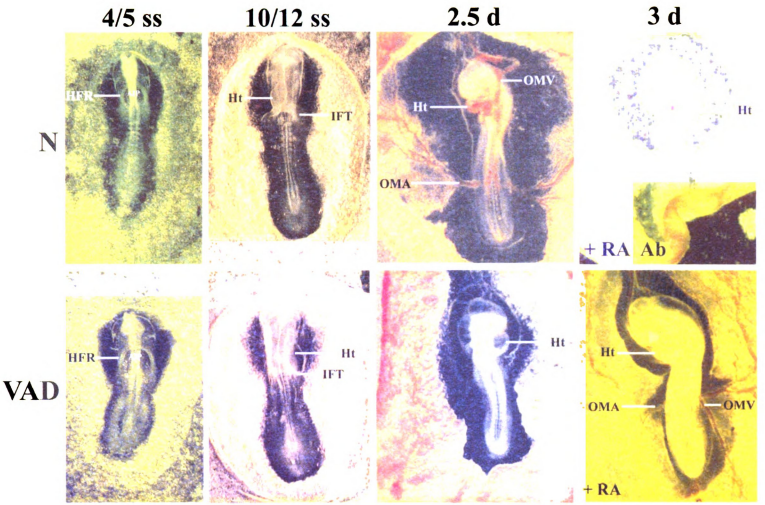

Figure 5.

Figure 5. Heart development in VAD quail embryos. There are no morphological differences observed in VAD and normal quail embryos at 4/5 ss. The bilateral heart primordia in the heart-forming region (HFR) line the anterior intestinal portal (AIP) and extend caudally as development continues. The heart-tubes fuse at the midline at 9 ss in the normal embryo, delayed to 10/11 ss in the VAD embryo. Heart (Ht) orientation is randomized in the VAD embryo (shown on left) and the inflow tracts (IFT) are closed by 12 ss. The Ht is a ballooned single-chamber at 2.5 d, in contrast to the two-chambered heart of normal embryos. VAD embryos lack omphalomesenteric veins (OMV) and omphalomesenteric arteries (OMA) to connect the embryo to the extraembryonic circulation. Injection of VAD embryos by 4/5 ss with biologically active retinoids, completely rescues cardiovascular development. If RA function is blocked in the normal embryo by injecting anti-atRA MoAb, cardiovascular abnormalities are observed consistent with the VAD phenotype

4.2 QH-1 monoclonal antibody is a marker for presumptive endothelial cells in normal and VAD embryos

The development of the vascular system requires differentiation of mesodermal cells in the HFR to endocardium (Coffin and Poole, 1988), as well as differentiation of mesodermal cells in the area vasculosa to angioblasts (Pardanaud, 1987). Inductive signals, i.e. growth factors, from the endoderm are required for the differentiation of mesodermal cells from both cell populations into presumptive endothelial cells (Wilt, 1965; Sugi and Lough, 1994). Mesodermal cells in the HFR region give rise to epimyocardial cells, as well as the endocardium; presumptive endothelial cells are distinguishable from premyocardial cells by the production of QH-1 and the expression of flk (Linask, 1992; Linask and Lash, 1993). In quail embryos this differentiation begins at the head-process stage and by the one-somite stage endothelial cells are detectable in the extraembryonic area and in the embryo in the area lateral to the somites (Coffin and Poole, 1988). Presumptive endothelial cells migrate from the extraembryonic area and the HFR towards the IFT, connecting at the IFT and then forming vessels. The VAD quail embryo lacks a vascular link between the extraembryonal and intraembryonic vascular networks at the IFT and at the dorsal aorta. We examined vascular development using QH-1 immunolocalization in normal and VAD embryos between 1 ss (early neurulation), when the first presumptive endothelial cells are detectable, and at 10/12 ss, when we observed the first morphological defects in the VAD cardiovascular development, i.e. the narrowing and closure of the IFT.

The first presumptive endothelial cells were observed at 1 ss in normal and VAD embryos in the extraembryonic area vasculosa and in the area pellucida, adjacent to the embryo (data not shown). QH-1 positive cells rapidly increased in both normal and VAD embryos between 1 ss and 4/5 ss. In the area vasculosa and the area pellucida presumptive endothelial cells assembled into loose polygonal-shaped networks, with strands of presumptive endothelial cells surrounded by large avascular areas (Figure 6). While no morphological differences between normal and VAD embryos were observed at this stage, VAD embryos had fewer QH-1 positive cells, most noticeable in the area pellucida (Figure 6). The polygonal networks of presumptive endothelial cells were incomplete and unorganized, with thin strands surrounded by large avascular areas. In normal and VAD embryos, presumptive endothelial cells lined the lateral edges of the AIP in the HFR. There were fewer QH-1 positive cells in the VAD embryo, judged by the lower intensity of fluorescence (Figure 6). A continuous strand of QH-1 positive cells of the dorsal aorta lined the somites by 7/8 ss in normal and VAD embryos; however, VAD embryos had fewer presumptive endothelial cells in the dorsal aorta and their organization was sporadic (Figure 6). By 7/8 ss vascular networks throughout the extraembryonic area had increased in normal and VAD embryos (Figure 6). However, the networks in the VAD embryo were sparse and there were fewer presumptive endothelial cells in the HFR and in the extraembryonal area compared with the normal embryo. Vascular networks in the normal embryo continued to coalesce into progressively thicker stands of presumptive endothelial cells. As

differentiation of mesodermal cells continues in the HFR, a rapid increase in presumptive endothelial cells in the HFR of the normal embryo was observed; strands of presumptive endothelial cells had formed to connect the intra- and extraembryonic vascular systems at the site that will later become the OMV (Figure 6).

In normal embryos by 10/11 ss presumptive endothelial cells coalesce into progressively thicker networks, surrounding small avascular areas. The endocardium of the heart is connected to the extraembryonic circulation via the OMV at the IFT. The formation of the OMA at the dorsal aorta completes the circulation (Figure 6). Prior to closure of the IFT in the VAD embryo only a few presumptive endothelial cells were seen in the posterior portion of the HFR and in the area pellucida (7/8 ss; Figure 6). At 10/11 ss there were relatively few QH-1 positive cells in the IFT of the VAD embryo, and the thin vascular networks present in the extraembryonal region at 7/8 ss had diminished even more. The OMV and OMA were absent in VAD embryos (Figure 6).

Administration of atRA to the VAD embryo before or during the retinoid-regulated developmental window completely rescued the abnormal presumptive endothelial cell patterning (Figure 6). In the rescued embryos extraembryonal vascular networks, OMV and OMA developed as in the normal embryo. Conversely, microinjection of the normal embryo with anti-atRA MoAb before the retinoid-regulated developmental window disrupted vascular patterning and the vascular pattern resembled that of the VAD embryo; the extraembryonic vascular networks were sparse and the OMV and OMA were disrupted.

Microinjection of normal and VAD control embryos with mouse IgG or vehicle had no effect on presumptive endothelial cell localization and vasculogenesis.

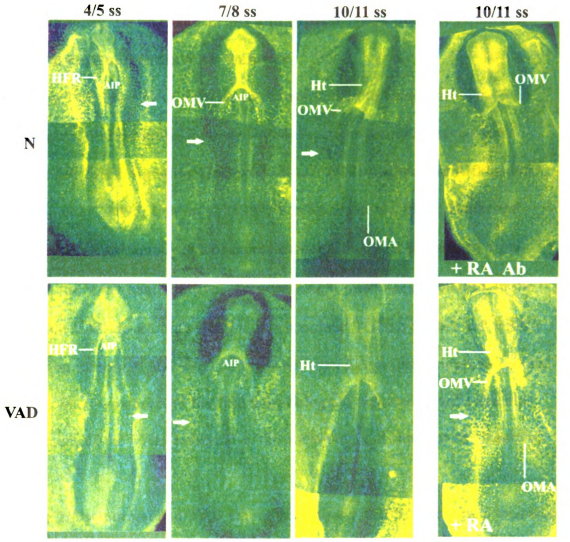

Figure 6. Composite pictures of normal and VAD embryos from 4/5 ss to 10/11 ss exposed to QH-1 antibody (ventral views). Endothelial cells as well as endothelial precursors are fluorescent (yellow). Angioblasts differentiate from mesoderm in the heart-forming region (HFR) and congergate along the lateral edge of the anterior intestinal portal (AIP). Simultaneously, angioblasts in the extraembryonic area differentiate from extraembryonic mesoderm and form vascular networks (arrow). Extraembryonic vascular networks connect with the heart (Ht) at the omphalomesenteric veins (OMV) and with the dorsal aorta lining the somites at the omphalomesenteric arteries (OMA). VAD embryos have fewer angioblasts, most notable in the IFT lining the AIP; the angioblasts are poorly organized and do not form OMV or OMA. The administration of atRA to VAD embryos completely rescues vascular development. Treating normal embryos with anti-atRA MoAb interferes with vascular patterning and results in a lack of connection of the heart to the extra-embryonic circulation at the OMV

4.3 Enhanced TGFβ2 transcripts and protein levels precede abnormal IFT development in VAD embryos

The TGFβ family of proteins have been linked to cardiovascular development. Furthermore, in several model systems retinoids have been implicated in TGFβ signaling (Jakowlew et al., 1992; Kojima and Rifkin, 1993; Mahmood et al., 1995; Imai et al., 1997; Yoshizawa et al., 1998; Tsuiki and Kishi, 1999). Additionally, we observed earlier that TGFβ expression was altered in the VAD quail embryo. To gain insight into TGFβ2 function in the normal and VAD developing embryo, we examined TGFβ2 protein and mRNA localization in normal and VAD embryos between 2/3 ss (early neurulation) and 10/12 ss, when we observed the first morphological defects in cardiovascular development of the VAD embryo, i.e. the narrowing and subsequent closure of the IFT.

In 2/3 ss normal and VAD embryos we found TGFβ2 transcripts expressed in the neural tubes, neural folds, Hensen's node, extraembryonal area, and in all cell layers of the HFR, most strongly in the mesoderm lining the AIP (Figure 8). VAD embryos expressed TGFβ2 transcripts more strongly in the Hensen's node and in the neural folds. TGFβ2 protein localization corresponded with TGFβ2 mRNA in normal and VAD embryo between 1/2 ss and 10/11 ss (Figure 7). At 4/5 ss, TGFβ2 transcripts increased in the HFR lining the AIP of normal and VAD embryos, and as neurulation continued the expression of transcripts continued to extend caudally through the neural folds (Figure 8). Expression of TGFβ2 transcripts and protein was increased in the posterior portion of the HFR

at this stage. Mesodermal cells in the HFR undergo differentiation into epimyocardial and endocardial cells, and heart-tubes begin to form in the normal and VAD embryo at 6/7 ss. At this stage, TGFβ2 transcripts in the HFR were expressed in the endoderm lateral to the AIP and the epimyocardium, which forms the lateral boundary of the HFR, throughout the IFT (Figure 8). Cross-sections through the HFR at the level of the IFT revealed TGFβ2 transcripts in all cell layers, including head mesenchyme, which in part forms embryonal blood vessels (Figure 8). Endothelial cells in the HFR form the OMV in the lumen between the endoderm and epimyocardium. TGFβ2 transcripts were expressed in the endoderm and the epimyocardium surrounding the lumen between the two cell layers (Figure 8) in normal embryos. VAD embryos expressed increased levels of TGFβ2 transcripts in the epimyocardium of the HFR, particularly the posterior portion of the IFT, as well as in the head mesenchyme (Figure 8). Prior to IFT closure, TGFβ2 expressing cells had almost completely filled the lumen between the endoderm and the epimyocardium in the IFT. TGFβ2 protein was also increased in the posterior IFT at this time (data not shown). Morphological closure of the IFT was observed at 10/12 ss in the VAD, at the same time when the expression of TGFβ2 transcripts and protein was increased in the IFT and caudal to the Hensen's node (Figure 7 & 8), as it was at 6/7 ss. Head mesenchyme continued to express increased levels of TGFβ2 mRNA. The lumen between cells expressing TGFβ2 in the epimyocardium and the endoderm in the IFT was becoming very narrow, and was completely closed in the posterior IFT region (Figure 8). Normal embryos expressed TGFβ2 transcripts and protein in

the endoderm and the epimyocardium of the IFT. At this developmental stage the OMV connect the embryo to the extraembryonic circulation and there are no TGFβ2 expressing cells in the lumen where the OMV form (Figure 8).

Administration of atRA to VAD embryos before or during the retinoid-regulated developmental window rescued TGFβ2 expression, i.e. TGFβ2 expression was down-regulated and resembled that of the normal embryo (Figure 8). TGFβ2 transcripts in the head mesenchyme were reduced and there was a large lumen between the epimyocardium and the endoderm. In contrast, microinjection of normal embryos with anti-atRA MoAb before the retinoid-regulated developmental window increased TGFβ2 transcripts, especially in the IFT (Figure 8). Blocking the RA with anti-atRA MoAb in these embryos resulted not only in increased TGFβ2 transcripts in the IFT, but also caused the lumen between the TGFβ2 expressing cells to narrow, and resemble the morphology characteristic of the VAD embryo. When normal and VAD control embryos were injected with mouse IgG or vehicle, TGFβ2 expression was not altered.

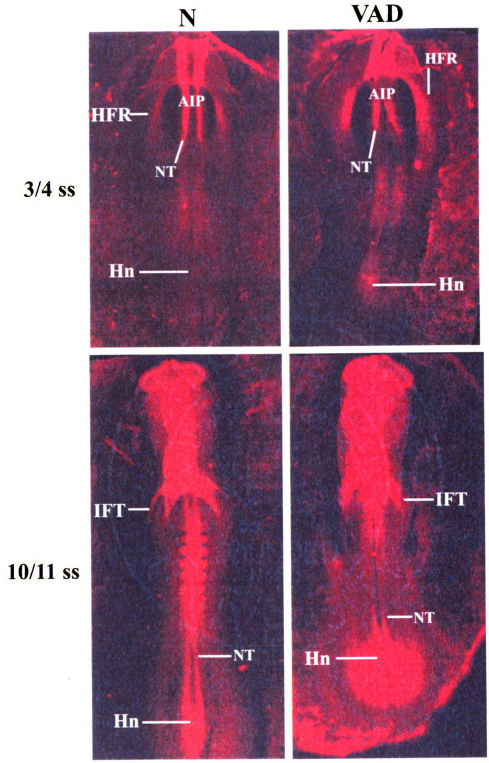

Figure 7.

Figure 7. Immunolocalization of TGFβ2 protein in normal and VAD quail embryos. At 3/4 ss TGFβ2 protein is ectopically expressed in the extraembryonic area. TGFβ2-specific immunofluorescence (red) is observed in the neural tubes (NT) of both normal and VAD embryos at comparable levels. TGFβ2-specific immunofluorescence is increased in the posterior heart-forming region (HFR) and caudal to the Hensen's node (Hn) in theVAD embryo at 3/4 ss. At 10/11 ss TGFβ2 is localized in the periphery of the developing inflow tracts (IFT) in the normal embryo and the absence of fluorescence at the site of the OMVs indicates that the IFT are open, while in the VAD embryo TGFβ2-specific fluorescence is increased in the IFT and the IFT are narrowed at the posterior ends. All views are ventral

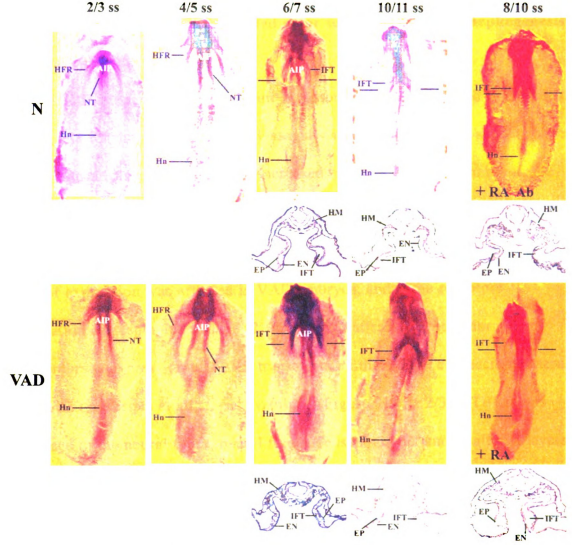

Figure 8. Expression patterns of TGFβ2 in whole-mounts of normal and VAD quail embryos, as well as in transverse sections through the inflow tract (IFT). Note the increased expression caudal to the Hensen's node (Hn) and in the heart-forming region (HFR) lining the anterior intestinal portal (AIP) of the VAD embryo beginning at the 4/5 ss. Increased expression in the IFT continues through 10/11 ss. The expression of TGFβ2 in the neural tubes (NT) of the VAD embryo preceded the observed expression in the normal embryo. As noted by transverse sectioning, at the level noted by the black horizontal lines bilaterally, TGFβ2 is expressed in all cell layers (6/7 ss). In VAD embryos, increased TGFβ2 trascripts were observed in the head mesenchyme (HM); cells expressing TGFβ2 filled the IFT between the epimyocardium (EP) and the endocardium (EN). Administration of atRA to VAD embryos reduced expression of TGFβ2 in the HM and the IFT, and the IFT were open. Treating normal embryos with anti-atRA MoAb resulted in increased TGFβ2 expression in the HM and the IFT, and the IFT were closed. All views are ventral.

4.4 TBRII transcripts are elevated prior to abnormal IFT morphogenesis in VAD embryos

TBRII is one the transmembrane receptors for the TGFβs and is the initial binding site for the mature ligand (Massague and Chen, 2000). As shown above, TGFβ2 transcripts and protein were increased in the IFT of VAD embryo. We examined TBRII, the principal receptor for TGFβ2, at the same developmental stages that we analyzed TGFβ2 expression (2/3 ss-10/11 ss), to determine if receptor expression was affected in VAD embryos, and if the receptors localization corresponded to TGFβ2 expression patterns. In both normal and VAD embryos expression of TBRII transcripts mimicked the expression pattern of TGFβ2 transcripts at all stages examined (Figure 9). At 2/3 ss TBRII was expressed in the neural tubes, neural folds, Hensen's node, and in all cell layers of the HFR, with increased expression in VAD embryo caudal to the Hensen's node at all stages examined. Expression of TBRII in the neural tubes extended caudally, as neurulation progressed. At 6/7 ss in the normal embryo TBRII was expressed in the epimyocardium and the endoderm of the IFT, surrounding the developing OMV; by 10/11 ss TBRII transcripts were observed in the epimyocardium and the endoderm surrounding the large lumen of the developing IFT (Figure 9). In the VAD embryo, TBRII transcripts were increased in the HFR at 4/5 ss and continued through 11 ss (Figure 9). Similarly, increased expression in the head mesenchyme and in the epimyocardium, as well as a narrowed lumen of the IFT, where the OMV typically connect to the heart, was

observed in the VAD embryo beginning at 6/7 ss and continued through the time of IFT closure at 10/11 ss.

Administration of atRA to VAD embryos before or during the retinoid-regulated developmental window completely rescued TBRII expression and the the IFT were open (Figure 9). Microinjection of anti-atRA MoAb into the normal embryo before the retinoid-regulated developmental window, increased TBRII expression, in particular in the IFT and resulted in a narrow lumen of the IFT, and an expression pattern characteristic of the VAD embryo (Figure 9). Administration of mouse IgG or vehicle to normal and VAD control embryos had not effect on TBRII expression.

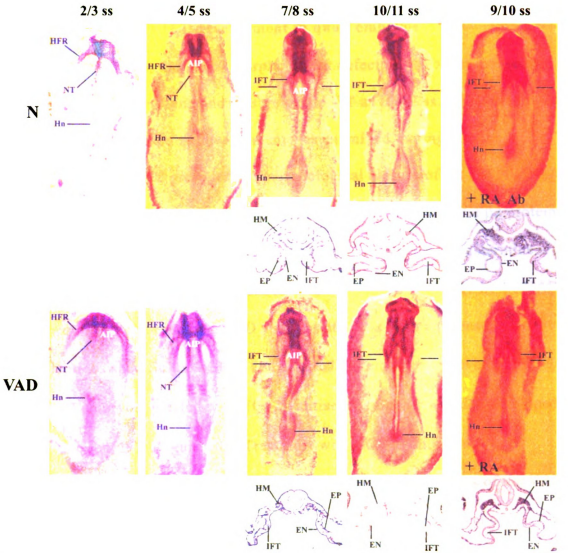

Figure 9. Expression patterns of TBRII in whole-mounts of normal and VAD quail embryos, and in transverse sections through the inflow tract (IFT). TBRII expression patterns correspond with TGFβ2 expression patterns in both normal and VAD embryos. VAD embryos have increased TBRII expression beginning at 4/5 ss and continuing through 10/11 ss. TBRII expression is increased in the IFT of VAD embryos beginning at 7/8 ss, prior to IFT closure. Transverse sectioning, at the level noted by the lack horizontal lines bilaterally, show a narrowing of the lumen between the EP and the EN in VAD embryos, and increased TBRII expression in the HM. Administration of atRA to VAD embryos reduced expression of TBRII in the HM and in the IFT, and the IFT were open. Treating normal embryos with anti-atRA MoAb resulted in increased TBRII expression in the HM and the IFT, and the IFT were closed. All views ventral. Anterior intestinal portal, AIP; endocardium, EN; epimyocardium, EP; head mesenchyme, HM; heart-forming region, HFR; Hensen's node, Hn; neural tubes, NT.

4.5 TGFβ2 mediates IFT development in quail embryos

As shown above, the initial morphological defect in VAD cardiovascular development is the narrowing and closure of the IFT at 10/12 ss. This effect was more apparent when vascular development was examined, noting that the lack of a vascular link at the IFT preceded the morphological closure of the IFT. We have also shown that VAD embryos have increased expression of TGFβ2 protein and mRNA, and TBRII mRNA in the IFT, prior to IFT closure. To determine if the elevated level of TGFβ2 was specifically involved in IFT closure in the VAD embryo, we injected normal and VAD embryos at 2/3 ss with antisense oligonucleotides specific for TGFβ2 and allowed the embryos to develop until 9-11 ss. VAD embryos injected with TGFβ2 antisense oligonucleotides developed normal, i.e. open, IFT (Figure 10). Treatment with TGFβ2 antisense oligonucleotides; however, did not normalize the other abnormalities of the VAD heart. TGFβ2 antisense oligonucleotides injection increased the level of presumptive endothelial cells in the IFT of VAD embryos resulted in formation of OMV that connected the heart to extraembryonic vascular networks; however, the networks were sparse and poorly organized (Figure 10, QH-1). TBRII expression was down-regulated in VAD embryos injected with TGFβ2 antisense oligonucleotides, specifically in the IFT (Figure 10). Similarly, TGFβ2 transcripts were decreased especially in the IFT, which resembled the open IFT of normal embryos with TGFβ2 expressing cells surrounding the OMV and the IFT developing normally, unlike the narrowed IFT of untreated VAD embryos (Figure 10).

Normal and VAD control embryos were injected with a random, non-specific antisense oligonucleotide. A small percentage (<5%) of both normal and VAD control embryos had abnormalities not associated with the VAD phenotype, possibly due to the toxic nature of degraded nucleotides; in general there was no effect on development. Embryos that exhibited these characteristic abnormalities were excluded from the sample group. Normal embryos injected with TGFβ2 antisense oligonuleotides at the concentration used in the current study developed normally.

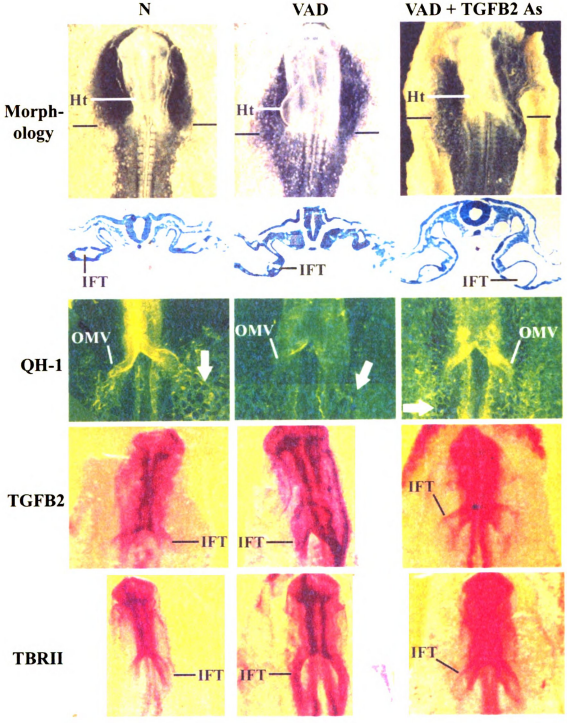
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Figure 10.

Figure 10. Blocking TGFβ2 transcripts in the VAD embryo by injecting antisense oligonucleotides to TGFβ2. The heart (Ht) of VAD injected with TGFβ2 antisense oligonucleotides was unaltered. Gross morphological examination as well as transverse sectioning at the level of the black lines located bilaterally, showed that the inflow tracts (IFT) remained open. VAD embryos injected with TGFβ2 antisense oligonucleotides, immunolabeled with QH-1, showed increased presumptive endothelial cells, specifically in the IFT, and the presence of omphalomesenteric veins (OMV). Vascular networks (arrow) in the extraembryonic area were better organized than in the VAD embryo; there were thin strands of presumptive endothelial cells extending from the extraembryonic area to the OMV; however, the vascular networks were not as organized, or robust as in normal embryos. Expression of TGFβ2 transcripts was specifically decreased in the IFT when VAD embryos were treated with TGFβ2 antisense oligonucleotides. Furthermore, treatment of VAD embryos with TGFβ2 antisense oligonucleotides had the secondary effect of decreasing TBRII transcripts in the IFT to levels comparable with the normal embryo. All views ventral

CHAPTER 5

DISCUSSION

It is well known from work in our laboratory and earlier studies that vitamin A is required for early avian development. In the VAD avian embryo, the earliest gross morphological abnormality is observed in the heart, which is a ballooned, non-compartmentalized single chamber structure randomly oriented and lacking IFT; therefore, without a link to the extraembryonal circulation.

The role of vitamin A in cardiovascular development is not understood. Our laboratory has addressed this question by examining the impact of VAD on cardiogenic genes, including heart asymmetry genes (Zile et al., 2000). However, no studies have been conducted on the role of vitamin A in IFT formation in the posterior heart region. The work in this thesis is the first to address specifically the question of how vitamin A regulates IFT formation, and to identify a gene, i.e. TGFβ2, as a downstream effector in the retinoic acid regulated pathway for IFT formation.

An association between RA signaling and the establishment of the posterior heart regions during early development is supported by work in our laboratory (Dersch and Zile, 1993; Twal et al., 1995; Kostetskii et al., 1999) and the observation that excess RA in the chick causes the domains of atrial myosin heavy chain (MHC1) expression to expand anteriorly, at the expense of ventricular MHC1 expression (Yutzey et al., 1994). Furthermore, retinaldehyde dehydrogenase, RALDH2, the earliest known RA synthesizing enzyme in the

mouse embryo, as well as RA response are initially restricted to the posterior regions of the developing mouse heart, suggesting a role of RA in the specification of cardiac inflow structures (Moss et al., 1998).

IFT development is a critical event in early embryogenesis and is a complicated process that requires differentiation of mesodermal cells in the HFR to epimyocardium and endocardium, migration of cells from the extraembryonic area towards the HFR and of cells from the HFR towards the extraembryonic vascular networks, which fuse together to form the basis of the circulatory system; proliferation and apoptosis are also a part of these processes. Clearly, many regulatory processes are involved, and it would be logical that the RA regulated pathway would involve multifunctional growth regulators. Therefore, TGFβs are likely candidates to be involved in RA-regulated cardiovascular development; furthermore, RA regulates TGFβ in many cell types.

Previous work in our laboratory using Northern blot, showed that TGFβ mRNA was up-regulated in VAD quail embryos (Kostetskii and Zile, 1993); however, these findings gave us no indications of where TGFβ was located in the developing embryo. If TGFβ2 were to be involved in heart development, we would expect it to be present in the HFR. There is only limited data regarding TGFβ2 expression in the heart during early cardiac morphogenesis. At later stages of cardiogenesis, after the heart has begun looping, TGFβ2 mRNA is expressed in the myocardial layer of the heart tube (Mahmood et al., 1995) and TGFβ2 protein is localized in all layers of the heart (Mahmood et al.,1992). In chicken embryos at stage 14, both TGFβ2 mRNA and protein were localized in

the myocardium and endothelium of the heart (Boyer et al., 1999; Barnett et al., 1994). Chicken embryos exhibit similar expression patterns to those in mice suggesting that TGFβ2 expression, and possibly function is conserved across species. Studies in mice have provided the only descriptive analysis of TGFβ2 expression during early cardiac morphogenesis showing that TGFβ2 mRNA is expressed in the promyocardium of 1-5 ss mice (Dickson et al., 1993). This result is consistent with our observation in both normal and VAD avian embryos, which express TGFβ2 mRNA in the heart splanchnic mesoderm during early neurulation. In mice embryos at 5-7 ss, TGFβ2 mRNA is expressed in the promyocardium of the vitelline veins and sinus venosus of the IFT, as well as in the foregut endoderm (Dickson et al., 1993). Similarly, we observed TGFβ2 mRNA in the epimyocardium of the IFT and in the foregut endoderm in stage-matched embryos. This expression pattern is conserved in the heart through the initiation of the heart beating at HH 10 or 8.5 d in the quail and mouse, respectively. Despite the early appearance of TGFβ2 mRNA, TGFβ2 protein is not detected until 8.5 d in the mouse and then it is expressed mainly in the cardiomyocytes (Dickson et al., 1993). In our current work we detect TGFβ2 early in neurulation, by 1/2 ss and continuing throughout the stages we examined (until 10/11 ss); however, recent work with chicken embryos using antibodies specific for active TGFβ2 protein did not find active TGFβ2 in the HFR at 10/11 ss (McCormick, 2001). However, in the same study, using ELISA, TGFβ2 was observed in whole-heart homogenates of 10/11 ss chicken embryos with 70% of the TGFβ2 in the active form. The levels of protein were shown to increase with

development, but the percentage of active TGFβ2 decreased with increased production of protein. Using whole mount embryos, we were able to show for the first time that TGFβ2 is located in the HFR and IFT of the developing normal avian embryo during the early stages of cardiac morphogenesis. Normally TGFβ2 is expressed at low levels, but the absence of vitamin A caused an over-expression of TGFβ2 throughout the embryo, but especially in the IFT. The concentration of antisense oligonucleotides used to block TGFβ2 in VAD embryos was sufficient to rescue IFT development, but it did not rescue other abnormalities associated with the VAD phenotype, suggesting a specific function in IFT development. Normal embryos injected with identical concentrations of antisense oligonucleotides exhibited no alterations in IFT development. This suggests that low levels of TGFβ2 specifically control normal, RA-regulated IFT development. Our findings are in agreement with the data showing that active TGFβ2 is present in the chick heart at the 10/11 ss (McCormick, 2001), and add support for the role of TGFβ2 in early cardiac morphogenesis.

Due to the complex nature of TGFβ2 signaling under physiological conditions, only limited data is available regarding the interaction of RA and TGFβ2 pathways in the heart. RA deficiency in mice results in down-regulation of TGFβ2 protein, while mRNA levels are unaltered (Mahmood et al., 1995), whereas treatment with excess RA decreases TGFβ2 protein in all embryonic tissues (Mahmood et al., 1992). In support of our present observations is the finding that knocking out RXRα results in increased TGFβ2 protein in the cardiac outflow tract of developing mice (Kubalak et al., 2002). The ability of

RA to decrease and normalize TGFβ2 mRNA expression in VAD quail embryos, as well as the alteration of normal expression of TGFβ2 when normal quail embryos are injected with anti-atRA MoAb, indicates that RA, the active form of vitamin A, regulates TGFβ2 expression during avian embryogenesis. However, the mechanism remains to be elucidated. Our overall findings are in line with those of others, indicating that RA interacts with TGFβ2 during embryogenesis suggesting that RA interaction with TGFβ2 is preserved across species.

TGFβ2 signaling is initiated via the binding of the ligand to TBRII, the primary receptor. TBRII mRNA is present in the developing chicken heart and its expression increases with developmental time (Barnett et al., 1994). However, TBRII localization in the developing heart has not been thoroughly studied. Our current work with the quail embryo is in agreement with the above studies in chicken as we find TBRII mRNA expressed in the embryonic heart and find this expression increased with advancing developmental stages. Our work here is the first to analyze the cellular expression of TBRII in early heart morphogenesis, and to examine it in relation to TGFβ2. TBRII mRNA is expressed in all layers of the developing quail heart and in the IFT, and is expressed in the same cells that express TGFβ2. The colocalization of TBRII with TGFβ2 is in agreement with the current understanding that TGFβ2 works through either autocrine or paracrine signaling mechanisms (Dickson et al., 1993; Boyer et al., 1999). As was the case with TGFβ2 transcripts, administration of RA to VAD embryos resulted in decreased TBRII transcripts in the IFT, while injection of normal embryos with anti-atRA MoAb increased

TBRII, supporting the role of RA in TBRII regulation. The ability of TGFβ2 antisense oligonucleotides to decrease the expression of TBRII transcripts in VAD embryos to normal levels, suggests that RA regulation of TBRII is a secondary effect of RA regulation of TGFβ2. In addition to decreasing TBRII mRNA expression, blocking TGFβ2 mRNA with antisense oligonucleotides enabled the IFT of VAD embryos to develop normally and to remain open, and also increased the number of presumptive endothelial cells in the IFT and the area pellucida of the VAD embryo. The specific role of TGFβ2 in RA-regulated cardiogenesis is restricted to the IFT at the developmental stages we examined, and is supported by the finding that the other phenotypical cardiac abnormalities of the VAD embryo were not affected by TGFβ2 antisense oligonucleotide blocking in VAD embryos. In the VAD embryo we see a decrease in presumptive endothelial cells (QH-1 positive) in the IFT, but generally there are more and likely, different cells that fill the openings. These results have led us to hypothesize that TGFβ2 inhibits endothelial cells in the inflow tracts during early cardiovascular development.

There are several possible explanations for the decreased number of presumptive endothelial cells in the VAD embryo: abnormal differentiation, decreased proliferation, increased apoptosis, and defective organization. We have shown that there are fewer presumptive endothelial cells in the VAD embryo and in the extraembryonic area from the beginning of vascularization and throughout the development of the cardiovascular system. We observed that presumptive endothelial cell numbers in VAD embryos are always below the level of that in normal embryos and that the addition of RA can rapidly

and drastically increased the number of presumptive endothelial cells; therefore, it is likely that RA mediates differentiation of mesodermal cells into presumptive endothelial cells, as well as mediating the proliferation of the newly generated presumptive endothelial cells. Our observation of fewer presumptive endothelial cells in the VAD embryo is supported by current work by LaRue and others indicating that vitamin A is required for the generation of the proper number of endothelial cells (LaRue et al., in preparation). In our studies we find that TGFβ2 expression is increased in the IFT of the VAD avian embryo; the increase of presumptive endothelial cells in VAD embryos when blocking TGFβ2 expression with antisense oligonucleotides, suggests that during normal embryonic development TGFβ2 restricts the generation of presumptive endothelial cells. As presumptive endothelial cells differentiate from mesodermal cells they undergo rapid proliferation. Several lines of evidence support the idea that TGFβ influences cell proliferation. Some studies show that TGFβ1 is a potent inhibitor of epithelial cell growth (Howe et al., 1993), while in others TGFβ1 has been shown to induce FGF-2, which results in fibroblast proliferation (Strutz et al., 2001). It is clear that TGFβ plays a role in cell proliferation; however, the direction and mechanism of regulation on cell proliferation needs to be determined empirically under different physiological conditions. It is possible that endothelial cell proliferation is decreased in VAD avian embryos, supported by evidence that has shown RA to stimulate proliferation of endothelial cell numbers (Melnykovych, 1981; Junquero, 1990; Lansink, 1998). In agreement with the role of TGFβ2 in both differentiation and proliferation of the vascular networks, is the observation that increased expression of TGFβ2 can inhibit vascular development at the

level of differentiation and proliferation in ocular tissue (Hayasaka et al., 1998; Zhao and Overbeek, 2001). All of the above observations support our hypothesis of a role of TGFβ2 as an growth regulator, limiting presumptive endothelial cell production in the RA-regulated formation of the IFT, possibly via decreased differentiation of mesodermal cells into presumptive endothelial cells and a subsequent decrease in presumptive endothelial cell proliferation.

TGFβ2 has been implicated as an inducer of cardiac muscle formation and differentiation. The spatio-temporal localization of TGFβ2, as well as other TGFβ isoforms, in the developing mouse embryo shows characteristics expected of a paracrine factor for cardiac muscle induction (Dickson et al., 1993). Specifically, TGFβ2 mRNA is present in cardiac precursor cells and in cells of the foregut epithelium, which is thought to have cardiac-inducing activity (Dickson et al., 1993), an expression pattern that we observed in both normal and VAD quail embryos. *In vitro*, TGFβ2 enhances cardiac muscle formation in cultured mouse embryonic stem cells, both in the rate of induction and in the number of muscle cells produced (Slager et al., 1993). While the above observations suggest a role of TGFβ2 in vitamin A-regulated cardiogenesis, there is no evidence that early cardiac muscle cell differentiation is regulated via a RA-TGFβ2 signaling pathway. Morphological examination of the heart and the IFT in VAD embryos does not reveal a thicker layer of epimyocardium surrounding the heart-tube and the IFT, as would be expected if the increased TGFβ2 expression in the VAD embryo IFT were to enhance cardiac muscle cell differentiation. Furthermore, examination of cardiac muscle cell-specific genes

in our laboratory has shown that expression of these genes is not affected in the VAD embryo (Kostetskii et al., 1999). While it is possible that TGFβ2 is involved in cardiac muscle induction, it seems unlikely that the retinoid-regulated physiological function of TGFβ2 in the early avian embryo is linked to this function.

Apoptosis is a critical process in vascular development needed for controlling cell populations and for remodeling. TGFβ2 and vitamin A have both been implicated in apoptosis; however, the mechanisms are unclear. Recent work has shown that reducing endogenous TGFβ prevents apoptosis of neurons in the developing retina of chick embryos (Dunker et al., 2001), while elevated TGFβ2 in RXRα knockouts enhances apoptosis in the cardiac outflow tract of developing mice (Kubalak et al., 2002). These findings suggest that TGFβ is capable of promoting apoptosis. A closer examination of the IFT of VAD embryos by sectioning the IFT of 10/11 ss embryos, stained to examine all cells, revealed that the lumen of the IFT in the VAD embryos were filled with cells. However, there were fewer presumptive endothelial cells in these IFT; thus TGFβ2 mediated apoptosis in the IFT would have to be a cell-specific signal for presumptive endothelial cells. Therefore, one cannot rule out an increased apoptosis of presumptive endothelial cells in VAD embryos, possibly mediated via excessive TGFβ2.

Another mechanism to explain the RA-TGFβ2 linked regulation of endothelial cell incorporation into the IFT would be due to an indirect effect of lack of RA on fibronectin. As mesodermal cells differentiate into presumptive

endothelial cells they migrate into strands before they form vessels (Pardanaud et al., 1987; Coffin and Poole, 1988; DeRuiter et al., 1993). Presumptive endothelial cells have been shown to migrate following fibronectin depostion in the extracellular matrix (Linask and Lash, 1986; 1990; 1992); moreover, it has been observed that fibronectin deposition is altered in VAD quail embryos (Linask and Zile, unpublished). Furthermore, antibody neutralization of TGFβ has been shown to reduce fibronectin deposition (Sanders et al., 1993), thus suggesting a possible role of TGFβ in endothelial cell migration. Since VAD quail embryos exhibit poorly organized vascular networks, it is possible that RA, via specific isoforms of TGFβ, mediates endothelial cell migration and organization.

In summary, RA-regulated cardiac IFT development is mediated via a TGFβ2 signaling pathway that most likely controls endothelial cell incorporation into the IFT structure, so as to form the link between the extraembryonic circulation and the heart. The specific target genes of TGFβ2, as well as the mechanism of RA regulation of TGFβ2 signaling, remain to be elucidated.

CHAPTER 6

FUTURE WORK

In this study we have provided evidence that links TGFβ2 to the development of the IFT and suggest that TGFβ2 inhibits endothelial cell differentiation; however, more research is needed to elucidate the role TGFβ2 in IFT development. As mesodermal cells differentiate, endothelial precursors express flk and myocardial precursors express N-cadherin. We have recently observed increased N-cadherin in the HFR of the VAD embryos. Moreover, N-cadherin is known to interact with TGFβ2 in many cell systems. A proposed study would be injecting VAD embryos at 1/2 ss with antisense oligonucleotides to TGFβ2 and collecting embryos sequentially from 30 min to 9 hr at which time the embryos will be approximately 7/8 ss. Then *in situ* hybridization can be done with biomarkers for endocardial and myocardial precursors and compare the results with normal and VAD embryos. If TGFβ2 signal initiated differentiation into muscle, then we would observe increased N-cadherin in the VAD compared to the normal and treatment with oligonucleotides would decrease the appearance of N-cadherin transcripts. To confirm the increase in presumptive endothelial cells we observed in VAD embryos injected with TGFβ2 antisense oligonucleotides by QH-1 immunolocalization, flk expression could be examined in a time course fashion. It would also be of interest to do double *in situ* hybridization with N-cadherin and TGFβ2 as well as flk and TGFβ2 to see if TGFβ2 is colocalized with a specific cell type, in order to gain insight into the

mechanism of action. We have recently linked RARα to IFT development. Blocking RARα in the normal embryo results in closure of the IFT. Examining the expression of TGFβ2 in these embryos could link TGFβ2 activity in IFT development with a specific RAR.

As we noted previously, we are not able to rule out other possible mechanisms of TGFβ2 regulation of presumptive endothelial cells during IFT development; however, there are several experiments that could be done to examine these mechanisms. We are currently developing an embryo culture system, which will simplify the study of migration and proliferation. Presumptive endothelial cell migration can be examined using DiI labeling of presumptive endothelial cells and examining their migration during IFT development to determine if and how endothelial cell organization is affected by vitamin A. BrdU staining of presumptive endothelial cells could give some insight into RA mediation of angioblast proliferation. It is possible that presumptive endothelial cells are differentiating from mesoderm and TGFβ2 is mediating apoptosis of these cells shortly after they differentiate. TUNNEL can be used to examine apoptosis at these early developmental stages after angioblasts differentiate.

TGFβs usually function as hetrotetramers. It will be important to determine the other TGFβ isoforms that function with TGFβ2 in mediating IFT development. The larger and more difficult question will be to try to determine what is upstream from TGFβ2 in the RA signaling pathway, as well as to determine the target genes of TGFβ2 in cardiac IFT development.

APPENDIX

Appendix 1. Additional procedures and composition of solutions

DNA loading buffer

- 0.25 % bromophenol blue
- 0.25 % xylene cyanol FF
- 15 % Ficoll in H_2O

Embryo acetone powder

- Dissect Embryos
- Grind in homogenizer
- Suspend in PBS (1 ml per 1 g tissue)
- Put tissue/salin on ice for 5 min
- Add 8 ml cold acetone per 2 ml of suspension and mix
- Incubate at 0°C for 30 min with occasional mixing
- Centrifuge at 10,000 g for 10 min (refrigerated centrifuge)
- Discard supernatant
- Add fresh cold acetone and allow to sit at 0°C for 10 min
- Centrifuge at 10,000 g for 10 min (regrigerated centrifuge)
- Discard supernatant
- Transfer pellet to filter paper and allow to dry
- Store at -20°C

Gelatin coated slides

- Place slides in 70% EtOH + 1 % HCl for 2 hr
- Rinse 10 times in ddH_2O
- Dip in Chromo-alum-gelatin solution
    - Heat 1L of ddH_2O to 65°C
    - Add 2 g gelatin
    - Dissolve and cool to 35-40°C
- Allow to drain
- Dip again
- Allow to dry overnight at 50°C

LB media/plates

- 95 ml ddH_20
- 1 g bacto-tryptone
- 0.5 g bacto-yeast
- 1 g NaCl
- Dissolve, pH 7.0
- Add ddH2O till 100 ml

- Autoclave
- Allow to cool
- For LB/Amp add Amp after cooled to working concentration of 50 μg/ml
- For LB plates add 1.5 g bacto-Agar before autoclaving
- Allow to cool, add Amp, pour plates, allow to harden, flame surface

Phosphate buffered saline (PBS)

- 8.0 g NaCl
- 1.44 g Na_2HPO_4
- 0.24 g KH_2PO_4
- 0.2 g KCl
- 1 L ddH_2O
- pH 7.4

RNA loading buffer

- 50 % glycerol
- 1 mM EDTA (ethylenediamine tetraacetate)
- 0.25 % bromophenol blue
- 0.25 % xylene cyanol FF

Toluidine Blue solution

- 1 g Touiding Blue
- 20 ml 95 % EtOH
- 15 ml ddH_2O
- 65 ml dioxane

Tyrode's solution

- 126 mM NaCl
- 22 mM dextrose
- 1 mM $MgCl_2$
- 4.4 mM KCl
- 20 mM taurine
- 5 mM creatine
- 5 mM sodium pyruvate
- 1 mM NaH_2PO_4
- 24.2 mM $NaHCO_3$
- 1.08 mM $CaCl_2$

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