UNIVERSITY OF LATVIA

FACULTY OF BIOLOGY

Inese Čakstiņa

Doctoral Thesis

Effect of retinoic acid on TGFβ2 protein and mRNA synthesis during early cardiovascular development *in vivo* and *in vitro*

Promotion to the degree of Doctor of Biology
Molecular Biology

Supervisors: *Dr.habil.biol.* prof. Indriķis Muižnieks

Ph.D.biochem. prof. Maija H. Zīle

Rīga, 2010

Annotation

Doctoral thesis title: "Effects of retinoic acid on TGFβ2 protein and mRNA synthesis during early cardiovascular development *in vivo* and *in vitro"*.

Clinical and basic research carried out during the last 50 years has established that vitamin A plays an important regulatory role in cell proliferation, differentiation as well as organogenesis in the developing embryo. Except for its role in vision, the functions of vitamin A are mediated by its physiologically active form, all-*trans*-retinoic acid (RA).

Vitamin A deficiency as well as excess dramatically influences the very early embryogenesis, especially cardiovascular development. Many genes, e.g. HoxB1, N-cadherin and transforming growth factor β2 (TGFβ2), that are involved in global regulation of embryonic development can be either up-regulated or down-regulated by vitamin A.

Heart is the first organ that develops during embryogenesis thus it is first to be under influence of different regulatory factors, including deficiency or excess of vitamins. Numerous animal models have shown that vitamin A deficiency induces cardiovascular abnormalities and impaired development that leads to early embryo lethality.

The use of the retinoid-deficient quail embryo *in vivo* model has revealed a time window at 4/5 somite stage (ss) when RA is essential for further development. Studies presented as part of this thesis demonstrate a major role for TGFβ2 in posterior heart development in the inflow tract region. It was determined that: 1) complete vitamin A deficiency (VAD) in quail embryos *in vivo* is paralleled by an extensive accumulation of TGFβ2; 2) the active form of vitamin A, RA, rescues heart development and down-regulates TGFβ2 mRNA synthesis as well as its protein production in the VAD quail embryo, and 3) suppression of TGFβ2 mRNA synthesis and protein activity in VAD embryos mimics the VAD rescue phenotype.

Here we describe the development of an *in vitro* avian model that can be used to elucidate the molecular basis of the observations described above. In primary cell cultures from the heart forming regions (hfr) of 4-7 somite stage chick embryos RA, supplemented in 10 nM and 100 nM concentration: 1) induces similar responses in the expression of TGFβ2 and HoxBI as were observed *in vivo*; 2) influences the expression of genes that are regulated by TGFβ2 through SMAD signaling pathway; and 3) induces the expression of the growth factor VEGF-A mRNA, one of the key factors for the differentiation of vascular endothelium. An antagonist of RA, Ro 41 5253, induces an upregulation of TGFβ2. We conclude that primary cultures of chick embryo hfr cells can be used to elucidate the molecular mechanisms underlying the down-regulation of TGFβ2 gene expression by RA.

Preliminary results from human embryonic stem cell derived cardiac progenitor cell line suggest a response similar to that observed in the cultures of chick hfr cells, i.e. TGFβ2 transcripts being down-regulated in the presence of excess of RA; however, TGFβ2 protein level was not altered in these human cells.

We conclude that there is an interaction between retinoic acid signaling and TGFβ2 synthesis and signal transduction; more data are needed to understand the mechanisms involved. The interaction may be explored in several disease model systems that are characterized by elevated levels of TGFβ2.

This work was conducted at Michigan State University (2003- 2005) and at the University of Latvia (2008- 2010).

Key words: retinoic acid, cardiovascular morphogenesis, quail embryo, transforming growth factor beta2 (TGFβ2), primary embryonic cell culture

Anotācija

Promocijas darba nosaukums: ”Retīnskābes ietekme uz TGFβ2 proteīna un mRNS sintēzi kardiovaskulārās sistēmas agrīnās attīstības stadijās *in vivo* un *in vitro*”

Pēdējo 50 gadu pētījumi ir parādījuši, ka A vitamīns ir nozīmīgs šūnu dalīšanās, augšanas, diferencēšanās, kā arī orgānu veidošanās modulators/regulators mugurkaulnieku embrioģenēzes laikā. Fizioloģisko procesu regulācijā A vitamīna aktīvā forma ir retīnskābe (RA).

A vitamīna trūkums, kā arī pārdozēšana izsauc smagus embrioģenēzes traucējumus, īpaši ietekmējot kardiovaskulāro attīstību. RA regulē vairākos attīstības procesos iesaistītu gēnu, piem., HoxB1, N-kadherīns, transformējošais augšanas faktors β2 (TGFβ2) darbību.

Sirds ir pirmais orgāns, kas veidojas embrioģenēzē, tāpēc tās attīstībā var labi novērot dažādu attīstību regulējošo faktoru ietekmi. Izmantojot vitamīna A deficītus (VAD) dzīvnieku modeļus, parādīts, ka tā izarisītās sirds un kardiovaskulārās sistēmas anomālijas un attīstības traucējumi noved pie embrija bojāejas.

Izmantojot VAD paipalu embriju modeli (*in vivo*), ir noskaidrots, ka viens no kritiskajiem laika posmiem, kad RA klātbūtne ir nepieciešama embrija normālai attīstībai, ir tieši 4-5 somītu stadija (ss). Šajā pētījumā parādīts, ka TGFβ2 piedalās sirds apakšējās daļas attīstībā, īpaši ieplūšanas traktu morfoģenēzē. Pilnīgs A vitamīna trūkums VAD paipalu embrijos *in vivo* ir saistīts ar paaugstinātu TGFβ2 proteīna uzkrāšanos. RA pievienošana VAD sistēmā normalizē sirds attīstību un pazemina TGFβ2 mRNS sintēzi, kā arī proteīna produkciju. Inhibējot TGFβ2 mRNS un proteīna sintēzi VAD embrijos, var atjaunot normālu sirds morfoģenēzi arī bez RA klātbūtnes.

Mēs esam izveidojuši *in vitro* modeli, kuru var izmantot, lai noskaidrotu molekulāros mehānismus, kas ir atbildīgi par RA iedarbību uz TGFβ2 gēnu ekspresiju un sirds morfoģenēzi. Cāļa sirdi veidojošā rajona primāro šūnu kultūrās, kas iegūtas no 4-7 ss embrijiem, un kultivētas barotnēs ar RA novērots, ka RA (i) izraisa globālo regulatoro gēnu TGFβ2 un HoxB1 ekspresijas atbildi līdzīgi kā *in vivo sistēmā,* (ii) ietekmē TGFβ2/SMAD signālceļa mērķa gēnu ekspresiju, (iii) stimulē vaskulārā endotēlija augšanas faktora VEGF ekspresiju. RA antagonista (Ro 41 5253) klātbūtnē TGFβ2 mRNS un proteīna sintēze ir paaugstināta. Izmantojot cilvēka kardiovaskulārās sitēmas priekštečšūnas, noskaidrots, ka RA, līdzīgi, kā putnu *in vivo* un *in vitro* sistēmās, kavē TGFβ2 mRNS sintēzi. Pieaugušu cilvēku sirds šūnās šāda atbilde nav novērota.

Pētījumā noskaidrots, ka pastāv mijiedarbība starp RA un TGFβ2 signāla pārneses ceļiem un TGFβ2 sintēzi, tomēr dziļākai mijiedarbības analīzei nepieciešami vairāk datu. Ar paaugstinātu TGFβ2 līmeni saistītos slimību modeļos būtu vēlams pārbaudīt mūsu novēroto RA inhibējošo ietekmi uz TGFβ2 sintēzi.

Darbs izstrādāts Mičiganas Štata Universitātē (2003-2005) un Latvijas Universitātē (2008-2010).

Atslēgas vārdi: retīnskābe, kardiovaskulārās sistēmas morfoģenēze, paipalu embriji, transformējošais augšanas faktors-beta 2 (TGFβ2), primārās embrionālo šūnu kultūras

Abbreviations

ADH	alcohol dehydorgenase
AKR	aldo-keto-reductase
AO	aorta
AP1	activating/activator protein 1
BCO-I	β,β-carotene-15,15-monooxygenase
BMP	bone morphogenic protein
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cM	centimorgan
CNS	central nervous system
co-SMAD	common SMAD / SMAD4
CPCs	cardiovascular progenitor cells
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
CRE/ATF	cAMP/activating transcription factor response element
CYP	cytochrome
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition/transformation
Endo	endothelial cell growth medium Endo-Grow (Millipore/Chemicon)
Erk	extracellular-signal-regulated kinases / classical MAP kinases
FBS	fetal bovine serum
FGF	fibroblast growth factor
FN / FN1	fibronectin
GTPase	guanosine triphosphate hydrolase
hfr	heart forming region
HH	avian development stages according to Hamburger and Hamilton, 1951
HPLC	high performance liquid chromatography
HUESC-24	human embryonic stem cell line 24
HUVEC	human umbilical vein endothelial cels
IFT	inflow tract
IGF	insulin-like growth factor
IL	interleukin
INF	interferon
JNK	c-Jun N-terminal kinase
kb	kilo base
KO-DMEM	knock-out DMEM

KO-SR	knock-out serum replacement
LAP	latency associated peptide
LRAT	lecithin:retinol acetyltransferase
LTBP	latent TGFβ binding protein
LTGFβ	latent TGFβ
LV	left ventricle
M-MuLV	Moloney murine leukemia virus
MAPK	mitogen-activated protein kinase
ME-OH	methanol
MEFs	mouse embryonic fibroblasts
MEKK1	MAPK/Erk kinase kinase 1
MH	Mad-homology domains
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
NEO	neogenin
OFT	outflow tract
p/s	penicillin/streptomycin
PAI-1	plasminogen activator inhibitor-1
PAR	polarity protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PFA	paraformaldehyde
PPAR	peroxisome proliferator-activated receptor
q	long arm of a chromosome
R-SMAD	receptor-regulated SMAD
RA / at-RA	all-trans retinoic acid
RALDH/Aldh	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP	retinol binding protein
RDH	retinol dehydrogenase
REH	retinyl ester hydrolase
Ro / Ro 41 5253	RA signaling antagonist
ROR	retinoid-related orphan receptor
RORE	retinoid-related orphan response elements
RPMI	Roswell Park Memorial Institute medium
RV	right ventricle
RXR	retinoid X receptor
SA	sinoatrial segment
SBE	SMAD binding element
SDR	short-chain dehydrogenase/reductase

SHH	Sonic Hedgehog signaling pathway
siRNA	small interfering ribonucleic acid
Smurf	SMAD-ubiquitination-regulatory factor
SP1	specificity protein 1
ss	somite stage
STAT	signal transducer and activator of transcription proteins
STRA6	Stimulated by Retinoic Acid gene 6
SV	*sinus venosus* (inflow tract)
TAK1	TGFβ-activated kinase 1
TF	transcription factor
TGFβ	transforming growth factor β
TGFβR / TBRII	transforming growth factor β receptor
THR	thyroid hormone receptor
TNF	tumor necrosis factor
TRAF6	tumor necrosis factor α receptor-associated factor 6
TTR	transthyretin
UTR	untranslated region
VAD	vitamin A deficient
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VSMC	vascular smooth muscle cell
α-MEM	α Minimal Essential Medium

Content

Introduction

All multicellular organisms have the same basic cellular mechanisms of cell movement, induction, determination, pattern formation, expression of homeobox genes, and programmed cell death during their development. Vertebrate cardiovascular system is the first physiological organ system that develops and forms during embryogenesis. Many of underlaying mechanisms are considered to be developmentally and molecularly conserved. Two basic processes are involved in cardiovascular development: one that drives morphogenesis (cell migration, proliferation and apoptosis), and one that is necessary for functional development (cell differentiation and determination of cardiovascular tissue). Both processes are regulated by the environment and signals within the embryo. These signals include vitamins, transcription factors, homeobox genes, cytokines, and different signaling molecules and pathways.

Vitamin A is crucial for vertebrate development and its insufficiency has profound effects on early embryogenesis. The active form of vitamin A, retinoic acid, RA, regulates many genes, including the ones involved in development. Animal models are an important tool to study early embryogenesis. Vitamin A deficient (VAD) animals develop various defects including an abnormal cardiovascular system that leads to embryo lethality. A cluster of RA regulated genes have been identified as participants in building the heart. RA regulation can be direct via binding to RARE sequences within target gene promoter or indirect through other unknown mechanisms.

Transforming growth factors β make up a large multifunctional cytokine superfamily that are major regulators of normal growth and development in vertebrates. The expression of TGFβs and their cell surface receptors is universal and widespread throughout the development. Several VAD animal model studies have linked TGFβ2 to cardiovascular development. Targeted disruption of TGFβ2 in mouse leads to various abnormalities, mostly related to heart defects. The above considerations gives the rationale for testing the hypothesis that RA is involved in the regulation of TGFβ2 synthesis and signaling during embryonic heart development and that TGFβ2 is among downstream effectors in a RA signaling pathway for these processes.

The aim of this study was to identify the mediators of RA effects on inflow tract formation during early cardiovascular development *in vivo* and *in vitro*.

To achieve this aim the following tasks were performed:

- histological characterization of normal and VAD quail embryo sections;
- determination of the expression level of novel RA-regulated genes potentially involved in heart morphogenesis;

- determination of the expression pattern and localization of TGFβ2 and TGFβRII in VAD quail embryos during early developmental stages;
- development of an *in vitro* system that could be used for further, detailed studies of the interaction mechanisms between RA and TGFβ2;
- determination of the effect of RA on TGFβ2 protein production and mRNA levels;
- investigation into the effects of RA on TGFβ/SMAD signaling target genes and on genes involved in IFT formation;
- preliminary studies of the effects of RA on TGFβ2 in human cells from various developmental stages (adult heart cells, umbilical vein endothelial cells, early cardiovascular progenitor cells).

1. Literature Overview

1.1. Structure, metabolism and biological activity of vitamin A

Vitamins are organic compounds required in small amounts by an organism and are involved in various fundamental body functions, including growth, maintenance of health and metabolism. Vitamins must be supplied by diet or as supplements because our bodies can not synthesize vitamins *de novo* (except for vitamin D). During 1900s, through experiments where animals were given different types of foods, scientists succeeded in isolating and identifying the various vitamins we know today. The fat-soluble vitamin A was discovered in 1913 by McCollum and Davis who fed purified diet with butter fat, egg yolk and lard or olive oil to a newly developed rat line (McCollum and Davis, 1913). Their subsequent findings demonstrated that fresh liver is rich in fat soluble-A (vitamin A) and water soluble-B and water soluble-C (McCollum et al., 1921). Soon it was discovered that yellow pigments of fruits and vegetables have similar nutritional effect. The first synthetic vitamin A (retinol) was made in 1947 by the Dutch chemists van Dorp and Arens (Olson, 1999).

Research in mid 20's and 30's of the last century showed that any imbalance, either deficiency or excess of vitamin A, dramatically changes differentiation of epithelial cells. Vitamin A plays an essential role in life of almost all chordates (invertebrate tunicates, lancelets and the vertebrates) in vision, in maintenance of epithelial surface, immune system, reproduction, embryonic growth and development. In humans the first response to insufficient uptake of vitamin A is altered ocular features (xerophthalmia) as well as a generalized impaired resistance to infection.

Maternal insufficiency of vitamin A during pregnancy in rats and pigs results in fetal death or offspring abnormalities with an obstructed development of cardiovascular and central nervous systems (Mason et al., 1935; Hale et al., 1937; cit. from Zile, 2010). The critical early developmental stage for vitamin A requirement in the early avian embryo coincides with the first 2-3 weeks of human pregnancy; heart formation may be severely compromised if maternal vitamin A intake is marginal or if there is interference with vitamin A function during pregnancy. While vitamin A deficiency is not prevalent in developed countries, the high incidence of vitamin A deficiency in developing countries may account for the increased incidence of heart malformations in these populations (Zile, 2010).

Excessive intake of vitamin A is associated with reduced bone mineral density and increased risk of hip fractures; excessive vitamin A intake during pregnancy is associated with embryonic malformations (Blomhoff and Blomhoff, 2006). The mechanism of action of retinoids is still poorly understood; many laboratories are working on different aspects of this important micronutrient.

The term *vitamin A* is a general term for retinol, the alcohol form of the vitamin. It is stored in the ester form, most commonly as retinyl palmitate (Fig.1.1). The aldehyde form, retinal, is important for the function and maintenance of vision. The physiologically most important form of vitamin A is its acid form, retinoic acid that functions at the gene level as a ligand for specific nuclear transcription factors that are involved in the regulation of fundamental biological activities of the cell. The term *retinoids* is currently used very broadly and includes both natural and synthetic compounds with vitamin A activity.

1.1.1. Structure, chemistry and metabolism of vitamin A

The body acquires vitamin A either as preformed vitamin A (usually as esters) from animal products (liver, kidney, egg, milk) or as provitamin A carotenoids (carrots, red peppers) that can be converted to vitamin A compounds in the body. All of these forms have beta-ionone ring (Fig.1.1) with an isoprenoid chain attached. The provitamin A, β-carotene, is the best source of vitamin A since it consists of two retinyl groups with β-ionone rings at both ends of the molecule (Fig.1.1). It is cleaved by β-carotene-15,15-dioxygenase in human small intestine into two molecules of retinal (Nagao, 2004). More than 600 carotenoids have been characterized; only 50 of them are consumed in the human diet and approximately 12 carotenoids account for most of the dietary intake. They are found in measurable concentrations in human blood and tissues and include: α-, β-carotenes, lycopene, lutein and β-cryptoxanthin (Crews et al., 2001; Voutilainen et al., 2006). Some (α-, β-carotenes), but not all (lycopene, lutein) carotenoids are able to produce vitamin A by an enzyme-induced pathway in the intestine/or the liver. Retinal is formed by central cleavage of the molecule and can be either reduced to retinol or oxidised to retinoic acid (Nagao, 2004).

Vitamin A as well as the provitamins A is fat-soluble; their absorption depends on the presence of adequate lipids and proteins in the meal. Deficiency of vitamin A can be due to intestinal disorders or disturbed lipid absorption. Only 3% of all carotenoids are absorbed and due to other metabolic reactions, only a fraction of carotenoids is converted to vitamin A; thus 12 μg of β-carotene is needed to obtain 1 μg of vitamin A activity.

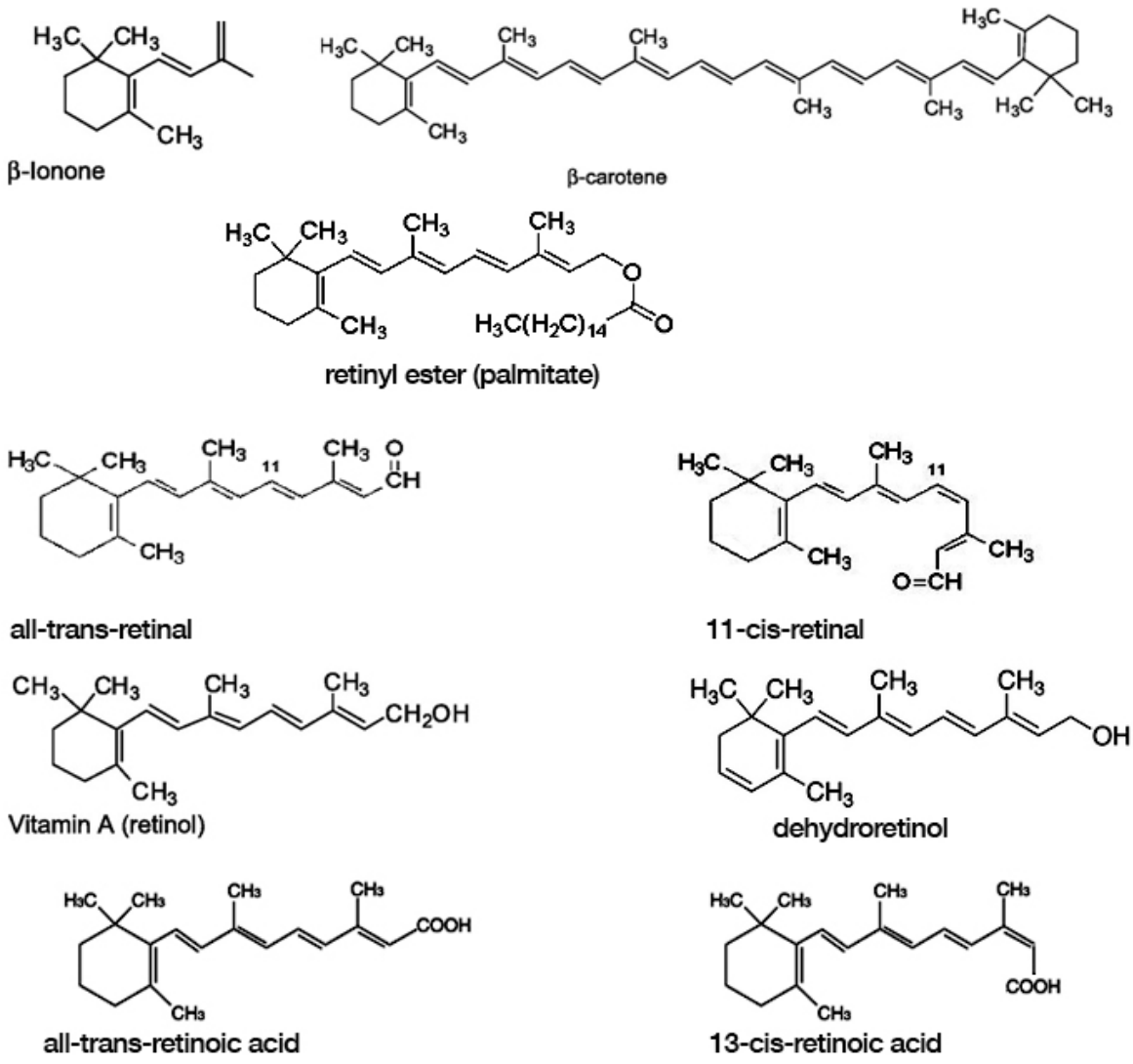

Figure 1.1. Structures of natural retinoids and their metabolites.

There are three main points about the fate of carotenoids in human upper gastrointestinal tract. First, carotenoids can not be absorbed while they remain embedded in their original vegetable matrices, emphasizing the role of stomach in absorption and digestion process. Second, carotenoids have to be solubilized in mixed micelles to be absorbed. Third, carotenoids are mainly absorbed by duodenal mucosal cells and enterocytes within small intestine by a mechanism involving passive diffusion, similar to that of cholesterol and the products of triglyceride lipolysis (Parker, 1996; Tyssandier et al., 2003). The rate of diffusion is likely determined by the concentration gradient between the micelle and the plasma membrane of enterocyte.

β-carotene obtained from diet in the enterocytes of the small intestine is first converted to the aldehyde form, retinal, that afterwards can be oxidized to retinoic acid or reduced to retinol (Fig. 1.2). Retinal is the major molecule after cleavage of β-carotene (Parker, 1996). Two types of cleavages are described in studies of β-carotene: central cleavage by β,β-carotene-15,15-

monooxygenase (BCO-I) and non-central cleavage (Napoli and Race, 1988; Parker, 1996). While central cleavage divides β-carotene molecule into half resulting in two all-*trans*-retinal molecules, non-central cleavage leads to the formation of two molecules of β-apo-carotenals with different chain length. The longer of the two molecules can subsequently be shortened enzymatically into retinoic acid or retinal. The enzyme responsible for the asymmetric cleavage, β,β-carotene-9,10-dioxygenase was cloned by von Lintig and coworkers (Blomhoff and Blomhoff, 2006). Retinal in the intestine is reduced to retinol. In human intestine, retinol is almost entirely esterified with 16- and 18-carbon fatty acids for transport to the liver in chylomicrons.

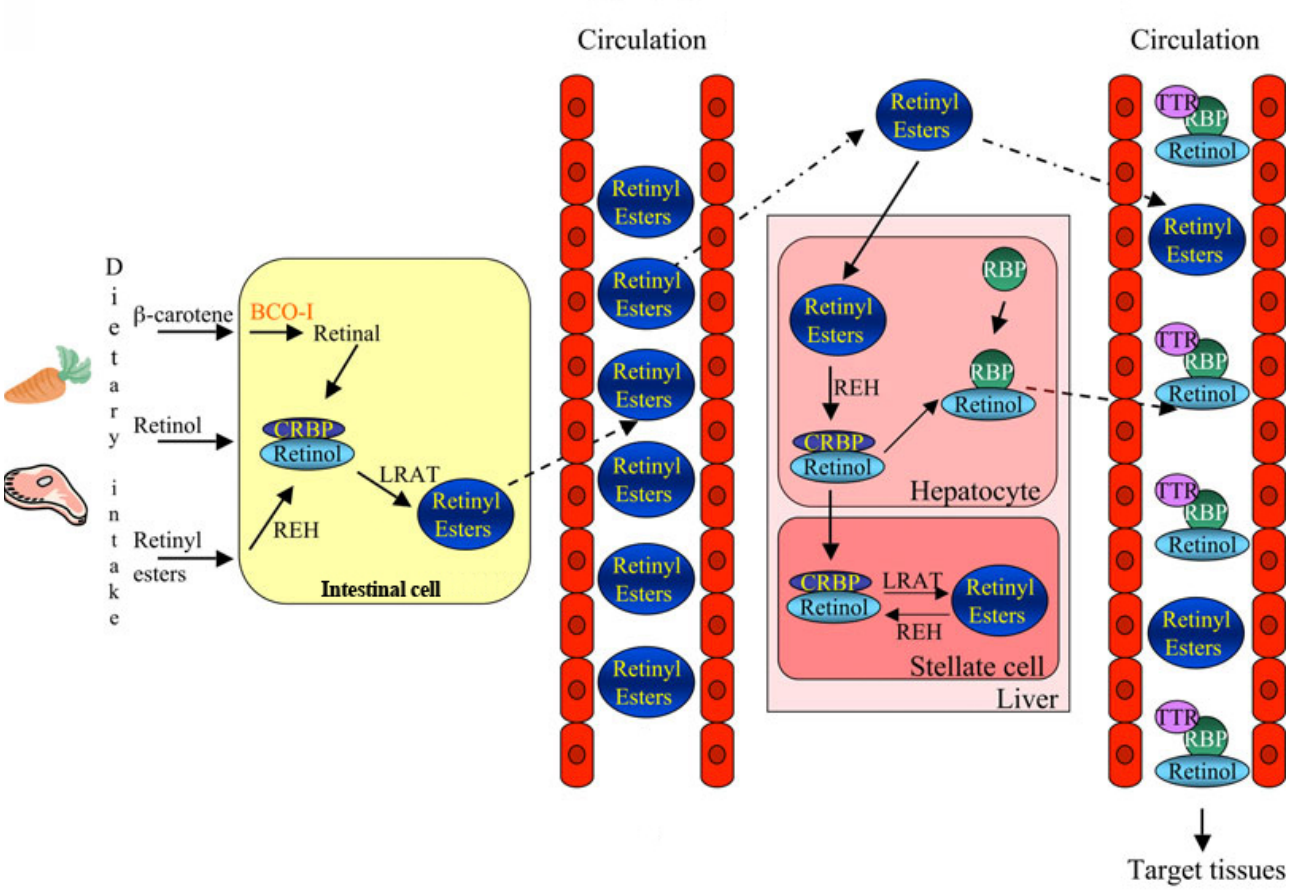

Figure 1.2. Metabolism of vitamin A and provitamin A (Theodosiou et al., 2010). BCO-I, β,β-carotene-15,15-monooxygenase; CRBP, cellular retinol binding protein; LRAT, lecithin:retinol acetyltransferase; RBP, retinol binding protein; REH, retinyl ester hydrolase; TTR, transthyretin.

Before the uptake by the enterocytes, dietary retinyl esters (e.g. retinyl palmitate) in the intestinal lumen are hydrolyzed to retinol by the pancreatic triglyceride lipase and intestinal brush border enzyme, phospholipase B (Harrison, 2005). The un-esterified retinol, when taken up by the enterocytes, is coupled with a specific binding protein, the cellular retinol-binding protein type II (CRBP-II). This complex serves as a substrate for re-esterification of retinol with long chain fatty acids (mainly palmitate) by lecithin:retinol acyltransferase (LRAT), while retinyl ester hydrolase

revert this process (Herr et al., 1993; Parés et al., 2008). The role of CRBP-II is to protect retinol from degradation and to direct it to LRAT (Batten et al., 2004).

Retinyl esters as well as uncleaved β-carotenes and small amounts of retinol are then incorporated into chylomicrons, the intestinal lipoproteins containing other dietary lipids, such as triglycerides, phospholipids, free and esterified cholesterol and apolipoprotein B (Roos et al., 1998; Harrison, 2005; Blomhoff and Blomhoff, 2006). These large lipoprotein complexes (100-2000 nm in diameter) are then secreted from the enterocytes into the intestinal lymph and transported via circulation for storage to liver or delivered to other tissues (Blomhoff et al., 1990). Under normal dietary conditions much of dietary vitamin A is absorbed via the chylomicron/lymphatic route. However, significant amounts of absorbed vitamin A are also secreted into portal circulation as unesterified retinol; it has been suggested that portal absorption is likely important in pathological conditions affecting secretion of chylomicrons (Harrison, 2005; Blomhoff and Blomhoff, 2006).

After reaching the liver, chylomicron-retinyl ester complex is hydrolyzed in the parenchymal hepatocytes, but retinol is re-esterified by LRAT and stored in perisinusoidal stellate cells, called Ito cells (Fig.1.2, Hirosawa and Yamada, 1973; Hoover et al., 2008). These cells have high levels of cellular retinol binding protein type I (CRBP-I) and LRAT, which are important for the storage of retinyl esters. Fifty to 80% of total body vitamin A in humans is stored in the liver stellate cells as retinyl esters. Some retinyl esters are stored outside the liver as lipid droplets in lung, kidney and intestine. This extra-hepatic storage is important for extra supply of retinol to tissues with high and constant vitamin A demand. The cells in the pigment epithelium of eye require vitamin A to biosynthesize 11-*cis*-retinal (Fig.1.1) for normal visual functions (Blomhoff et al., 1990; Blomhoff and Blomhoff, 2006).

When required, stored retinyl esters in the liver are hydrolyzed, and the free retinol binds to its serum transport protein, the retinol binding protein (RBP). Most of retinol:RBP in circulation is associated with the thyroid hormone transport protein, transthyretin (TTR), and released into circulation to prevent elimination by kidney (Buxbaum and Reixach, 2009). This complex delivers retinol (together with thyroid hormone) to target tissues (Fig.1.2). Liver is the main site of RBP synthesis; RBP recycles extensively between the liver, plasma and extra-hepatic tissues (Blomhoff and Blomhoff, 2006). The normal reserve of retinyl esters in the liver ensures a steady blood plasma retinol level in spite of fluctuations in daily vitamin A intake. Retinol is released from stellate cells to maintain constant physiological concentration in the plasma of 2 μmol/L. In addition to retinol and retinyl esters, other retinoids are found in plasma at nanomolar concentrations, such as all-

trans-retinoic-acid (RA); the concentration of RA in plasma and other body fluids is 5-10 nmol/L (De Ruyter et al., 1979; Eckhoff and Nau, 1990; Roos et al., 1998).

Liganded RBP (holo-RBP) delivers retinol to target tissues, where retinol undergoes multiple enzymatic reactions. The existence of a specific cell surface receptor for RBP was demonstrated in studies from 1970s, but only recently this receptor was identified and found to be regulated by *STimulated by Retinoic Acid* gene 6, STRA6 (Kawaguchi et al., 2007). STRA6 binds specifically to RBP and mediates retinol uptake from holo-RBP. Interestingly, STRA6 is broadly expressed in the murine embryo, but in the adult its expression becomes more restricted to stellate cells, peritubular pigment epithelial cells and embryonic carcinoma cells (Redondo et al., 2008; Theodosiou et al., 2010).

It is generally believed that active vitamin A metabolites are synthesized within target cells. Retinol from plasma serves as a major source for this synthesis. Other lipoproteins containing retinyl esters, retinol and carotenoids as well as locally stored retinyl esters in lipid droplets in target cells or their neighboring cells also contribute to synthesis of active metabolites (Blomhoff and Blomhoff, 2006). The most active form of vitamin A is RA (Kastner et al., 1995; Ross et al., 2000; Balmer and Blomhoff, 2002; Blomhoff and Blomhoff, 2006; Niederreither and Dollé, 2008). The synthesis of this metabolite occurs in a two step reaction: oxidation of retinol to retinal and oxidation of retinal to RA. Cells are able to catalyze the reverse reaction from retinal to retinol, but are not capable of retinoic acid conversion back to retinal (Napoli, 1993).

The first step of vitamin A cellular metabolism is hydrolysis of retinyl esters to retinol, then the reversible oxidation of retinol to retinal, catalyzed by cytosolic alcohol dehydrogenases (ADHs) and microsomal retinol dehydrogenases (RDH) of the short-chain dehydrogenase/reductase (SDR) superfamily (Fig.1.3, Parés et al., 2008).

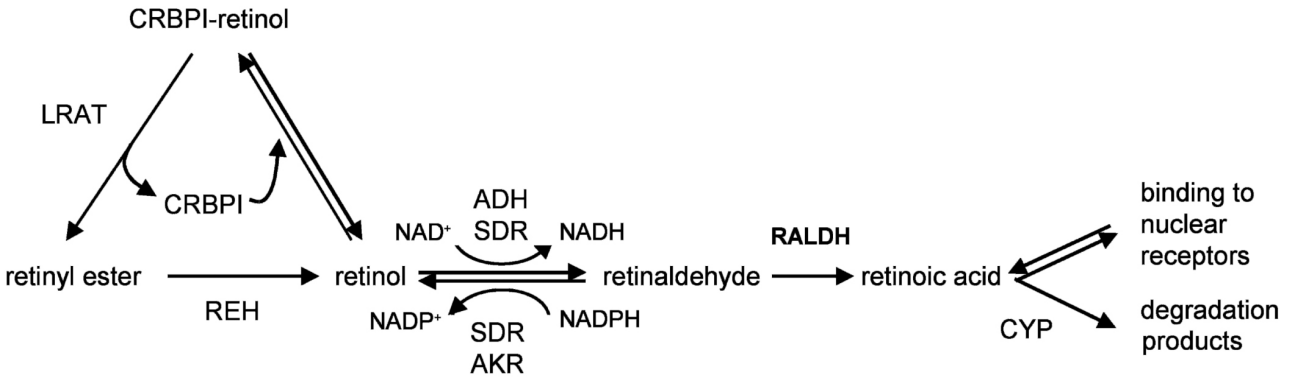

Figure 1.3. Schematic overview on cellular retinoid metabolism (Parés et al., 2008). ADH, alcohol dehydrogenase, AKR, aldo-keto-reductase, CRBPI, cellular retinol binding protein type I, CYP, cytochrome P450, LRAT, lecithin:retinol acyltransferase, RALDH, retinaldehyde dehydrogenase, REH, retinyl ester hydrolase, SDR, short-chain dehydrogenase/reductase.

These enzymes use only free retinol. Retinaldehyde reductase activity has been detected in members of the aldo-keto reductase (AKR) superfamily (Parés et al., 2008). Retinol metabolism is catalyzed by the widely expressed ADH3 as well as the tissue-restricted ADH1 and ADH4. ADH1 and ADH3 function in liver to stimulate degradative retinol turnover to retinal which prevents retinol toxicity, whereas ADH4 and ADH3 in peripheral tissues oxidize retinol to retinal to generate RA for signaling to nearby cells (Parés et al., 2008). While ADHs are cytosolic, SDRs are microsomal. The oxidation of retinal to RA is a cytosolic process thus ADH functions in RA synthesis; the retinal produced by SDRs may also be required for processes other than RA synthesis (Theodosiou et al., 2010).

Retinal is irreversibly oxidized (Fig.1.3) to RA by various retinaldehyde dehydrogenases. Vertebrates have three RALDHs of the Aldh1A class (called RALDH1 or Aldh1A1, RALDH2 or Aldh1A2, and RALDH3 or Aldh1A3) that are essential for embryonic development as demonstrated in knock-out studies (Niederreither et al., 2001). This oxidation is generally believed to be the rate-limiting step in the biosynthesis of RA (Perlmann, 2002; Blomhoff and Blomhoff, 2006). Data from all RALDH knock-out studies indicate that different mechanisms are used for RA synthesis by different cells (Niederreither et al., 2001). RALDH1 is expressed at high levels in the dorsal retina of embryos and in several adult epithelial tissues, but knock-out studies demonstrate only minor expression in dorsal retina suggesting that this enzyme is not essential for RA synthesis, but is instead involved in the catabolism of excess retinol (Duester et al., 2003). RALDH2 on the other hand is essential for RA synthesis, especially during embryonic development. Animal studies demonstrate the localization of RALDH2 in mesenchymal cells including trunk mesoderm, proximal limb bud, lung bud mesoderm as well as heart (Ulven et al., 2000; Niederreither et al., 2001; Duester et al., 2003). RALDH3 is expressed in mouse and chick retina, lens and olfactory pit, as well as in ureteric buds and surface ectoderm over the developing forebrain. Mice RALDH3 knock-outs die within the first 10h after birth due to defects in nasal development (Dupé et al., 2003).

Retinoic acid can be synthesized directly from β-carotene in intestine, liver, kidney and lung. This process does not require prior oxidation of β-carotene to retinol (Napoli and Race, 1988; Nagao, 2004). Thus β-carotene and other carotenoids may be a source of RA, especially in humans who are capable of accumulating high concentrations of tissue carotenoids (Blomhoff and Blomhoff, 2006).

After biosynthesis, RA can bind to the cellular RA binding proteins (CRABP-I and CRABP-II) and migrate to nucleus (autocrine signaling) for receptor binding, or be transported to a nearby cell (paracrine signaling). In the absence of ligand, CRABP-II is found in the cytosol, but in the presence of RA it translocates to the nucleus, where the complex associates directly with retinoid receptor complex and mediates ligand transfer from the binding protein to the receptor (Dong et al., 1999).

Since RA in higher doses is toxic, a balance between biosynthesis and degradation is necessary for the control of its levels in cells and tissues. Catabolism occurs mainly through enzymes of the CYP26 family and is initiated by hydroxylation of the C4 or C18 position of the β-ionone ring of RA leading to oxidized metabolite forms of RA such as 4-hydroxy-RA and 4-oxo-RA. The cytochrome P450 enzyme (CYP26A1) was first cloned in zebrafish showing the ability of degradation of RA (White et al., 1997). This enzyme was afterwards cloned in the human, mouse, rat and chicken (Blomhoff and Blomhoff, 2006). It is localized at high levels in liver, duodenum, colon and placenta as well as in some regions of brain (Lampen et al., 2001). The proximal upstream promoter region of CYP26A1 gene contains a functional RA response element (RARE) sequence, therefore transcripts are induced by RA, demonstrating the mechanism through which CYP26A1 gene can sense the elevated concentrations of RA and regulate the oxidative metabolism accordingly (Lampen et al., 2001; Hernandez et al., 2007). Some of the degradation products, such as 4-hydroxy-RA, 4-oxo-RA, 18-hydroxy-RA and retinol- and retinoic acid -β-glucuronide are reported to be biologically active (Reijntjes et al., 2005; Barua and Sidell, 2004). The water-soluble RA metabolites are excreted in bile and urine (Blomhoff and Blomhoff, 2006). In several studies of catabolism of retinoids by analyzing urinary, biliary and fecal metabolites other, more polar metabolites have been detected, including decarboxylation, epoxidation and isomerization products (Blomhoff and Blomhoff, 2006).

1.1.2. Biological activity of vitamin A

The major biological functions of vitamin A are in cell growth and differentiation. The requirement of vitamin A for normal embryonic and fetal development is known from many nutritional studies (Zile, 2004; 2010). Vitamin A also is essential for the formation and maintenance of many body tissues, such as skin, bone and vasculature, as well as for vision and immune function (Collins and Mao, 1999). In humans, the role of vitamin A extends to adulthood regulating fertility,

maintaining normal vision and preventing neoplastic growth and neurodegenerative disease (Niederreither and Dollé, 2008).

Deficiency of vitamin A (VAD) leads to blindness and infectious diseases, whereas less severe VAD results in retarded growth and iron deficiency anemia (Theodosiou et al., 2010). Conversely, excess dietary vitamin A can result in toxicity to the liver, central nervous system (CNS), the muscle-skeletal system, internal organs, and skin (Collins and Mao, 1999). Hypervitaminosis A can lead to reduced mineral bone density, and increased risk for hip fracture, and to malformations of the developing embryo.

Vitamin A is converted to its most active compound, RA, through which it regulates embryonic development and organogenesis, tissue homeostasis, cell proliferation, differentiation and apoptosis at the gene level. The developmental roles of RA have been studied using a range of vertebrate models including zebrafish, amphibia, chickens, quail and mice, demonstrating RA morphogenic actions. RA is one of the first morphogens identified (Ide and Aono, 1988; Mitrani and Shimoni, 1989). Morphogen is a substance that is active in pattern formation, the spatial concentration or activity of which varies and to which cells respond differently at different concentrations. RA is a rapidly diffusing signaling molecule that can control gene expression through the activation of specific nuclear receptors thus specifying cell identities.

The best understood function of vitamin A is its non-genomic role in vision. The human retina has two distinct photoreceptor systems: the rods, containing rhodopsin, which can detect low-intensity light, and the cones, containing iodopsin, which can detect different colors. In the retinal pigment epithelium retinol can be converted to 11-*cis*-retinal that is transferred to the photoreceptor cells and works more like co-factor. This aldehyde form of vitamin A, 11-*cis*-retinal, is the prosthetic group on both visual proteins. The mechanism of vitamin A action in vision is based on the ability of the vitamin A molecule to photoisomerize (change shape when exposed to light). Thus, in the dark, low-intensity light isomerizes the rhodopsin prosthetic group, 11-*cis*-retinal, to all-*trans*-retinal. The changes in shape lead to a change in electrical charge generating an electrical signal that is transmitted via the optic nerve to the brain, resulting in visual sensation as light perception.

Reproduction includes many processes: oogenesis, ovulation, spermatogenesis, fertilization, implantation, embryonic development, placental functions and fetal development. In mammalian experimental models, retinol is required at the placental stage. Once it is inside the cells, it is converted to the active form RA that can then regulate differentiation and growth processes at gene level. The function of vitamin A for all reproductive processes is not clear.

1.1.3. General classification of genes regulated by retinoic acid

The pleiotropic effects of vitamin A are attributable to a multitude of RA-linked transcriptional pathways involved in numerous cellular processes, including embryonic development. Retinoic acid is among the most important signaling molecules in vertebrate ontogenesis.

Different studies throughout the decades have gathered important information regarding vitamin A and its functions at the genome and proteome level in organisms. More than 500 genes have been reported as regulatory targets for RA signaling. However, not all of them have RAREs that are necessary for direct ligand-receptor heterodimer binding within their promoter region. Thus it is clear that many genes can be regulated indirectly by RA as the result of interaction with intermediate transcription factors or via other mechanisms.

The known RA-regulated genes have been categorized and a classification table constructed consisting of four major categories (i.e 0-4) of genes based on approximately 2000 papers covering 532 genes (Balmer and Blomhoff, 2002). This table summarizes the experimental evidence available for each target gene and gives the degree to which the evidence supports or disproves the fact of direct regulation, at least in the cellular context.

RA can influence gene and protein functions through several pathways. The predominant, classical pathway is the one where RA, when liganded to the RAR/RXR hetereodimer, directly interacts with its RARE, its DNA response element in the target gene. It must be clarified that genes in category 3 are as much RA target genes as those in category 0. There are 27 genes in category 3 that are unquestionably regulated directly by RA. Category 2 contains 105 genes which can be affected less than 6 h after administration of 1μM or less RA, but indicators for direct regulation (i.e. RARE) are lacking. As for most of the 133 genes in category 0, there are no data that these genes are regulated directly through the classical RA pathway. Some of these genes are doubted to be regulated at the transcriptional level. The remaining category 1 contains 257 genes that are regulated by RA, but it is still not clear if their regulation is direct or indirect. The authors of this gene table conclude that most of the RA-regulated genes are up-regulated (approx. 300) while fewer genes are down-regulated (approx. 120). However, the direction of transcriptional regulation varies depending on cell type or developmental stage of the organisms. Furthermore, there are no observations on a systemic regulation of genes by RA and it is not possible to arrange the RA-regulated genes in clusters (Balmer and Blomhoff, 2002; 2005).

1.1.4. Mechanism of action of RA

With the exception for its role in vision, the pleiotropic effects of vitamin A are mediated by its physiological active form, RA, which functions as the ligand for it specific nuclear receptors, retinoic acid receptors (RARs) that form heterodimers with the retinoid X receptors (RXRs). Almost all nuclear receptors have two binding domains: DNA binding domain in their N-terminal and ligand binding domain in the C-terminal (Mangelsdorf et al., 1995; Xiao et al., 1995).

The RARs were discovered independently in 1987 by two groups, i.e. the Evans (Giguere et al., 1987) and Chambon (Petkovich et al., 1987) laboratories. There are three subtypes of RARs, i.e. RARα, RARβ and RARγ. Their amino acid analysis has higher conservation among different species than within given species. Each subtype has at least two isoforms generated by different promoters (P1 and P2), that have RARE. These isoforms can be transcribed using P1 or P2, also alternative splicing can result in different isoform, but these isoforms differ only in their N-terminal region (Germain et al., 2006a; de Lera et al., 2007). There are two major isoforms for RARα (α1 and α2) and for RARγ (γ1 and γ2) and four isoforms for RARβ (β1, β2, β3 and β4). RARβ1 and RARβ3 are initiated at the P1 promoter whereas RARβ2 and RARβ4 are initiated at the P2 promoter (Germain et al., 2006a). Subsequently, in 1990's, a new subfamily of receptors, the retinoid X receptors (RXRs) and their subtypes, RXRα, RXRβ and RXRγ were cloned (Mangelsdorf et al., 1990; 1992). There are two major isoforms for each RXR subtype, e.g. RXRα1 and RXRα2 (Germain et al., 2006b). The RARs and RXRs are expressed differently throughout the development and their separate roles still remain unclear (Xiao et al., 1995; Germain et al., 2006 a,b).

The RARs function mainly as heterodimers with RXRs, i.e. as RAR/RXR and act as ligand-dependent transcriptional regulators by binding to the specific RARE DNA sequences found in the promoter region of retinoid target genes. The highest affinity ligand for RARs is all-*trans*-RA whereas for RXR another isoform, 9-*cis*-RA appears to be the best activator, but the physiological role of this RA isoform is still unclear (Germain et al., 2006b). RXRs also regulate transcription in heterodimeric complexes with the vitamin D receptor, VDR and the thyroid hormone receptor, THR, and are also necessary for efficient binding to DNA of the peroxisome proliferator-activated receptors (PPARs), that are involved in multiple aspects of fatty acid metabolism, (Ziouzenkova and Plutzky, 2008).

RAREs consist of two or more direct repeats of directly repeated hexameric degenerate copies of (A/G)G(G/T)TCA or more relaxed (A/G)G(G/T)(G/T)(G/C)A half-site motifs separated usually by five or two nucleotides (Balmer and Blomhoff, 2005). RAR/RXR heterodimers are able to regulate transcription not only from DR5 (direct repeat with a five-nucleotide spacer), but also from DR2 and DR1 elements (Durand et al., 1992). RXR homodimers recognize RXREs that have only one nucleotide spacing in between direct repeats (De Luca, 1991; Xiao et al., 1995).

RARs carry out their functions through the recruitment of different regulatory proteins, i.e. the co-activators or co-repressors which are not exclusive for RARs but are capable to bind with other DNA-binding proteins (Xiao et al., 1995). The binding of RA to its receptors releases the co-repressors or results in the recruitment of co-activators. Receptor/co-activator complexes induce histone acetylation resulting in chromatin decompression and an increase in target gene expression. Opposite process takes place when the receptor associates with a co-repressor leading to histone deacetylation and chromatin compaction and a decreased gene expression (Jetten, 2009; Rochette-Egly and Germain, 2009). Deletion or silencing of the expression or of the production of RARs often results in an effect similar to that seen in vitamin A deficiency (Cui et al., 2003).

Data about RA regulated genes have been summarized in a table (Balmer and Blomhoff, 2002). Interestingly, more than 100 target genes are not regulated through the classical RAR/RXR binding to the RAREs. There is a wide range of genes that are regulated by RA and its receptors, but that lack the RARE sequence within the promoter. This has resulted in the search for potential different mechanisms, one of them suggesting protein-protein interaction. It has been well documented that an activated (liganded) RAR /RXR interacts with the activating protein 1, AP-1, a heterodimeric transcription factor composed of fos- and jun-related proteins involved in cell proliferation mechanisms (Pfahl, 1993; Zhou et al., 1999). This interaction leads to inhibition or alteration of the expression of genes that are regulated by AP-1, e.g. the transforming growth factor beta2 (TGFβ2) thus resulting in negative regulation (Noma et al., 1991; Blomhoff, 1997). For example, the anti-AP-1 activity of RXRα as well as of RAR isoforms has been observed to inhibit three different AP-1 controlled promoters, i.e. in TGFβ1, collagenase and cFos (Salbert et al., 1993). Moreover, the data from Evans lab (Schule et al., 1991) show that RA/RARα can form a non-productive complex with c-Jun providing the mechanism by which RA can limit cell growth and malignant progression (Schule et al., 1991; Rochette-Egly and Germain, 2009). An example of such mechanism is the RA anti-cancer activity in the treatment of acute promyelocytic leukemia patients. Since the addition of all-*trans*-retinoic acid to this therapy, approximately 72% of patients can achieve complete remission (Degos and Wang, 2001; Germain et al., 2006a,b). This number has

been increasing when all-*trans*-retinoic acid treatment was used together with arsenic trioxide, as up to 93% of these patients achieved 5-year overall survival rates; the survival rates are approaching 100%. The question remains, why this treatment does not have such an effect in leukemia subtypes (Petrie et al., 2009).

Another transcription factor, the specificity protein SP1, has been reported to associate with the RAR/RXR heterodimer and to bind to the SP1 specific site, called GC box element on target gene promoter sequences (Bouwman and Philipsen, 2002; Cheng et al., 2008).

Ongoing research is devoted to discovering and describing other signaling mechanisms, especially those related to different isoforms of RARs. There are data that link RARs to RNA binding, as well as to translational regulation, phosphorylation and regulation of chromatin stability (see Rochette-Egly and Germain, 2009).

The nuclear receptor discovery of the late 80's lead to identifying many so called "orphan receptors" that have a similar structure to other known receptors, but their endogenous ligands are unknown. The class of retinoid-related orphan receptors consists of three main isoforms, α, β, γ, that also regulate gene transcription by binding to DNA response elements (ROREs) consensus AGGTCA preceded by 6-bp A/T rich sequence in promoter region of target genes (Jetten, 2009). The data from non-denaturing mass spectrometry and scintillation proximity assays provide evidence that RA and several other retinoids can bind to the RORβ and RORγ, but not to the RORα ligand binding domain, reducing RORβ-mediated transcriptional activity and inhibiting RORγ-mediated transactivation (Stehlin-Gaon et al., 2003; Wada et al., 2008; Jetten, 2009). However, other data show that RA can regulate RORs also through its classical signaling pathway involving binding with the RAR/RXR heterodimer and regulating ROR gene expression. Further studies are necessary to determine whether RA is a genuine physiological ligand for these ROR receptors (Jetten, 2009). The RORs are involved in many developmental processes such as cerebral development and retina development, and are also critical regulators of thymopoiesis and development of several secondary lymphoid tissues (Jetten, 2009).

1.1.5. Role of RA in development

Many nutritional studies with animals have shown the requirement of vitamin A for normal embryonic and fetal development as vitamin A deficiency or excess can lead to serious developmental abnormalities. Vitamin A in its active form as RA, can affect almost every organ and tissue if the embryo is treated with RA at critical time points during development (reviewed in Zile,

2001; 2004; 2010). Vertebrate embryos have the ability to metabolize vitamin A and generate at-RA but underlying cellular and molecular regulatory mechanisms that are affected by endogenous RA are not fully understood. It is important to understand the regulatory mechanisms since RA is a strong morphogen and affects cell survival, proliferation, differentiation and organogenesis in developing embryos. Various developmental studies have been carried out *in vivo* (zebrafish, *Xenopus laevis*, chicken, quail and mice) to examine the role of vitamin A during embryogenesis and fetal development. These studies demonstrated that RA and the RARs regulate the expression of many developmental genes resulting in a wide range of functional alterations that include axial patterning, regional patterning of the central nervous system, neurogenesis, cardiovascular development, limb development and the formation of other organs (Niederreither and Dollé, 2008).

Various strategies have been used to study retinoid signaling in early embryogenesis. One of the strategies is the gain-of-function approach and is used mostly to study the effects of RA excess in specific regions at specific developmental stages. Many studies use concentrations significantly above endogenous levels, and thus they may not reflect the physiological functions of vitamin A. Another strategy is the loss-of function approach involving the blocking of RA signaling, using antisense oligonucleotides to RA metabolism genes, or blocking the metabolisms of RA with monoclonal antibodies against RA (Romeih et al., 2003; Zhou et al., 1991).

Among other approaches is the nutritional approach where retinoid signaling is switched off by the generation of vitamin A-deficient embryos (Dersch and Zile, 1993), or the knock-out approach by the generation of transgenic mice via mutations or knock-outs of the RAR genes since their expression is widespread throughout different developmental stages (Luo et al., 1996; Ross et al., 2000; Rochette-Egly and Germain, 2009). Altering RA metabolism is another important and often used approach to examine the the physiological role of vitamin A in *in vivo* embryo models. This involves the interference with metabolism by knocking out genes of RA generating enzymes, such as Raldh2 (Niederreither et al., 2001; Ribes et al., 2005) and Raldh3 (Molotkova et al., 2007) or the genes of RA degrading enzyme Cyp26 (MacLean et al., 2007) or the generation of combination knock-outs of RAR/Cyp26 or Cyp26/Raldh2 (Niederreither and Dollé, 2008). However, there has always been a concern about incomplete silencing of RA signaling (discussed in Dupé and Lumsden, 2001), or in case of RARs, that they do not provide a complete and clear VAD phenotype due to receptor redundancy (Iulianella and Lohnes, 2002) and the involvement of RXRs in other signaling pathways (Ross, 2000). Nevertheless, these studies have provided important insights into the various pleiotropic effects of vitamin A and have demonstrated the critical role of RARs in vertebrate development.

At this time, the only model showing the absolute essentiality of vitamin A for embryonic development is unambiguously demonstrated in the vitamin A-deficient (VAD) avian embryo. The completely VAD embryo develops gross abnormalities in the cardiovascular and central nervous systems and trunk that end with early embryo lethality. Significantly, these embryos can be rescued leading to normal development by administration of the physiological ligand for RARs, all-*trans*-retinoic acid, or its precursor, retinol, at a certain time point during a critical stage in early development (Dersch and Zile, 1993; Zile, 2004; 2010).

Crucial developmental decisions in the vertebrates take place during the neurulation stage (4-5 somite stage, ss), which is inaccesible to *in vivo* manipulations in mammals but easily accessible in the avian embryo. This developmental stage in the avian coincides with 2-3 weeks of human development. Although the RA synthesis system and its receptor transcripts are present before this crucial window, it is not until the time of initiation of RA requirement that the embryo first requires vitamin A (Kostetskii et al., 1998; Cui et al., 2003; Zile, 2004). Studies in the Zile lab have demonstrated that the RARα2, RARγ, and RXRα are the essential and required receptors to transfer RA signals at the 4-5 ss for avian embryonic development and survival (Romeih et al., 2003).

There are no master regulators in the heart development, but instead many signaling pathways are involved in the complex interactions to form the cardiovascular system from embryonic mesoderm and endoderm. Among the early signaling molecules are the superfamily of transforming growth factors that include activins A and B, TGFβ2, TGFβ3, BMP2 and BMP4 (Sporn and Roberts, 1992; Kingsley, 1994). This family of genes is also involved in cardiac left/right determination as well as in pericardiac cell differentiation into cardiomyocyte lineage and vascular development. Strong evidence for TGFβ2 involvement in cardiac morphogenesis is provided by knock-out mice models that develop multiple developmental defects including those in the heart similar to those reported in the offspring of VAD rat (Wilson et al., 1953) and with the defects observed in RAR and RXR knock-out models (Mendelsohn et al., 1994; Sucov et al., 1994).

Several transcription factors such as the GATAs-4/5/6, and the homeobox genes Nkx2.5, HoxB1 and Msx-1 have been linked to cardiac commitment and differentiation (Lyons, 1996). Interestingly, Nkx2.5 gene knock-outs have abnormal heart development and absence of heart looping, similar to the defects observed in VAD quail embryo. However, the expression of Nkx2.5 is not altered in VAD quail embryos (Kostetskii et al., 1999).

GATA genes regulate several cardiovascular developmental processes. GATA4 is required to allow cells to respond to cardio-inductive substances; it mediates BMP signaling by combined

action with Nkx2.5 (Pikkarainen et al., 2004). The posterior part of the heart tube has high levels of GATA4 transcripts (Jiang et al., 1998). This observation lead to the examination of the expression of GATA4 in VAD quail embryo, which does not form the inflow tract, the complex structure in posterior heart tube that links embryonic heart to the extra-embryonic vasculature. GATA4 is severely diminished in the heart-forming regions of the VAD embryo. In contrast, cardiac muscle-specific gene expression involved in cardiomyocyte differentiation is not regulated by RA signaling *in vivo* in VAD quail embryos (Kostetskii et al., 1999).

Msx1 is known to be involved in heart morphogenesis (Chen et al., 2007); it has a RARE in its promoter region thus is sensitive to a direct regulation by RA/RAR/RXR (Mangelsdorf et al., 1995). The expression of Msx1 is down-regulated by endogenous, physiological retinoids *in vivo* during early avian embryogenesis (Chen et al., 1995).

N-cadherin, one of the cell adhesion molecules involved in cell assembly and rearrangement during embryogenesis is crucial for cardiovascular development (Linask, 2003; Kostetskii et al., 2005). Studies of N-cadherin expression in VAD quail embryos demonstrate its involvement in vascularization and heart looping, but not in regulation of heart asymmetry. Furthermore it was shown that N-cadherin is negatively regulated by RA; since its promoter region does not contain RARE, this regulation may be mediated by Msx1 (Romeih et al., 2009).

RARs and RXRs are also important transcription factors linked to heart development as demonstrated in knock-out studies that report a wide spectrum of heart abnormalities (Ross et al., 2000). Studies with VAD quail embryos demonstrate that RARα2 has a distinct role in cardiac inflow tract formation, whereas RARγ regulates cardiac L/R asymmetry and looping (Romeih et al., 2003).

In the VAD quail embryo, initially the embryonal and extra-embryonal vascular systems appear to develop normally; however, subsequently, the vascular networks are not maintained, the *vitelline* veins do not form and the endocardial tubes close, and thus there is no cardiac inflow tract (Zile, 2004; 2010). There is abnormal vasculogenesis in the VAD quail embryo correlated to diminished vascularization and this can be rescued by administration of vitamin A to the VAD embryo (Dersch and Zile, 1993; Zile, 2004). Unpublished work (Zhou and Zile, 2010) suggests that the extracellular matrix protein fibronectin (FN) is regulated by RA; this is of interest since treatment of embryos with an antibody to FN disrupts cell migration and causes abnormal heart development (Linask and Lash, 1988). Furthermore FN knock-out mice develop abnormal but beating heart and defects in vasculogenesis (Watt and Hodivala, 1994).

While vitamin A via RA regulates many events during embryonic and fetal development, it has a crucial role during early embryonic development. There is a relatively limited time window when presence of the vitamin A active form, RA, is absolutely essential for normal cardiovascular development as well for initiating early developmental gene pathways that affect many aspects of the subsequent embryogenesis. Understanding how physiological levels of RA regulate different signaling pathways and target genes in various tissues at different time points is a challenge for further research.

1.2. Transforming growth factors

The transforming growth factors betas (TGFβ) make up a large multifunctional extracellular cytokine superfamily of structurally similar proteins that are major regulators of normal growth and development in vertebrates (Roberts and Sporn, 1992; Chang et al., 2002; Azhar et al., 2003; 2009; Hoover et al., 2008). There are more than 35 members of this superfamily in vertebrates; they include TGFβs, Activins, Inhibins, bone morphogenic proteins (BMPs), growth differentiation factors (GDFs), and the asymmetry molecules Nodal and Lefty (Kingsley, 1994; Hogan, 1996). The TGFβ superfamily members as ligands can form homo- and hetero-dimers that bind to and activate two types of serine/threonine kinase receptors, which stimulate regulatory SMAD proteins to localize from cytoplasm to nucleus where they function as transcriptional regulators (Heldin et al., 1997; Whitman and Raftery, 2005). TGFβs can signal through multiple pathways (to be described later in this section). TGFβ superfamily signaling is regulated at various levels, e.g. at extracellular binding and processing of TGFβs ligands and intracellular interactions of the receptors (Chang et al., 2002; Wu and Hill, 2009; Moustakas and Heldin, 2009). Abnormalities in TGFβ signaling lead to various defects and are linked to congenital heart disease, predisposition to cancer and cardiovascular pathogenesis (Euler-Taimor and Heger, 2006; Jiao et al., 2006; Gordon and Blobe, 2008). The three isoforms of TGFβs 1, 2 and 3 exhibit a variety of proliferative, inductive and regulatory functions (Sporn and Roberts, 1992) and have been linked to cardiovascular development (Azhar et al., 2003; Mercado-Pimentel and Runyan, 2007; Azhar et al., 2009). They have complex signaling patterns in the cardiovascular system (Euler-Taimor and Heger, 2006) and are potent regulators of vascular development (Goumans et al., 2002; Bobik, 2006) and hematopoiesis (Larsson and Karlsson, 2005).

In most of the scientific literature the term “TGFβ” is used loosely, without distinguishing between different isoforms, mainly to describe the functions of TGFβ1 since this isoform is the one

most studied. TGFβ2 is the isoform most strongly linked to heart morphogenesis (McCormick, 2001; Austin et al., 2008). This is supported by observation in TGFβ2 knock-out mice that exhibit abnormalities in heart development, which are the most prominent among other defects as compared to those in lung, craniofacial, limb, eye and ear development (Dünker and Krieglstein, 2000; Molin et al., 2004). TGFβ2 is also involved, in epithelial-mesenchymal cell transformation (EMT) during avian heart development (Romano and Runyan, 2000; Jiao et al., 2006; Mercado-Pimentel and Runyan, 2007; Azhar et al., 2009).

It is well known that in many cells RA regulates TGFβ signaling (Mahmood et al., 1992; Yoshizawa et al., 1998) with TGFβ2 being the most sensitive isoform to tissue retinoid levels (Glick et al., 1991). Inhibition of TGFβ signaling by endogenous RA is required for mouse lung development (Chen et al., 2007). On the other hand, RA-induced negative regulation of TGFβ2 leads to altered EMT (described later), that is needed for septation in atrioventricular canal during heart morphogenesis (Romano and Runyan, 2000; Mercado-Pimentel and Runyan, 2007).

1.2.1. Molecular structure of TGFβs, LTBP and their receptors

The three isoforms of TGFβ genes expressed in vertebrates are localized on different chromosomes. TGFβ1 occupies part of chromosome 19 (19q13.1) in human, chromosome 7 (7 6.5cM) in mouse and chromosome 13 in chicken. TGFβ2 isoform lies within chromosome 1 (1q41) in humans, and in chromosome 1 (1 101.5cM) in mouse and chromosome 3 in chicken. The isoform TGFβ3 is located in chromosome 14 (14q24) in humans, chromosome 12 (12 41.0cM) in mouse and chromosome 5 in chicken. The chicken TGFβ2 gene consists of 7 exons and has several splicing variants (data from PubMed Entrez-Gene database).

There are multiple TGFβ2 mRNA transcripts or approximately 3.0, 4.1, 5.1 and 6.5 kb in humans (Madisen et al., 1988) and mouse (Miller et al., 1989). In chicken there are three TGFβ2 transcripts of size 3.9, 4.3 and 8 kb that are expressed differentially in developing chick embryos where 8-kb form is expressed at higher levels while in chicken chondrocytes and fibroblasts, 3.9 and 4.3 kb forms are dominant (Jakowlew et al., 1990). Isolation and sequencing of human and mouse TGFβ2 cDNAs have revealed multiple potential poly-adenylation signals (Madisen et al., 1988; Miller et al., 1989). It has been suggested that differential utilization of these signals in the 3'-untranslated region (UTR) could generate the heterogeneity in transcript size (Webb et al., 1988). Although it has been reported that RA increases the levels of TGFβ2 mRNA and protein in several cell types by a post-transcriptional mechanism (Glick et al., 1989), little is known of mechanisms

regulating transcription of TGFβ2 gene *in vivo* and *in vitro*. Collective studies of TGFβ2 gene structure in human and mouse cells suggest that regulation of TGFβ2 expression occurs at multiple levels, including transcription, alternative exon splicing, and alternative utilization of poli A+ sites and post-transcriptional stabilization of mRNA (Noma et al., 1991).

S1 nuclease protection analysis identified a transcriptional initiation site 1357 nucleotides (nt) 5' of the methionine initiation codon (ATG) and TATA box was identified 30 base pairs (bp) from this transcriptional start site as is characteristic of many eukaryotic promoters (Noma et al., 1991). Several AP1, AP2, CRE/ATF and SP-1 like DNA consensus sequence elements have been also identified surrounding the transcription initiation site (Noma et al., 1991). Studies with human TGFβ2 promoter demonstrate high levels of promoter activity within the DNA constructs containing nt between -508 to +63, whereas sequences between -778 and -508 nt modulated this promoter activity in a manner which was dependent upon the used cell line, suggesting the regulation of TGFβ2 being dependent of cellular background (Noma et al., 1991). The first study to identify a DNA element CRE/ATF necessary for transcription of TGFβ2 gene was conducted by O'Reilley and colleagues where they show that TGFβ2 promoter is regulated, in part; through a CRE/ATF binding site located 74 nt 5' of the TATA box (O'Reilly et al., 1992). The consensus element of activating transcription factor (CRE/ATF) was originally identified as a segment of DNA that was able to confer cAMP responsiveness to the phosphoenolpyruvate carboxykinase (GTP) gene and has subsequently been shown to confer cAMP responsiveness to a variety of genes (Roesler et al., 1988). This CRE/ATF sequence is necessary for basal gene expression. Comparison of TGFβ2 gene in human and in chicken revealed that although the coding regions are remarkably conserved between species, only the TAT and CRE/ATF elements are conserved in the promoter regions (Burt et al., 1991).

The TGFβ family ligands, like typical secreted signaling molecules, are translated as pre-proproteins that consist of signal peptide, pro-domain followed by the mature domain (Fig.1.4) (Padgett et al., 1997; Massagué, 1998). An amino-terminal signal peptide targets the precursor molecule to a secretory pathway whereas a pro-domain of variable length is involved for correct protein folding, dimerization of subunits and regulation of factor activity (Kingsley, 1994; Massagué, 1998).

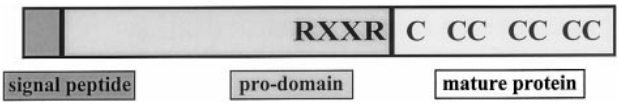

Figure 1.4. Structure of TGFβ family precursor molecules that are synthesized as pre-proproteins with signal peptide at N-terminal region targeting the molecules to the secretory pathway, followed by pro-domain and mature domain. Dibasic cleavage at RXXR motif leads to release of carboxy-terminal domain that homo- or hetero-dimerizes producing biologically active molecule (Böttner et al., 2000).

Cleavage of pro-domain from the mature domain occurs at dibasic cleavage sites, RXXR motifs. During this process the carboxy-terminal fragment of 110-140 amino acids is released. Biologically active molecules are generated by homo- (characteristic for TGFβ2) or hetero-dimerization of these carboxy-terminal domains (Böttner et al., 2002). Six to nine conserved cysteine residues in the mature domain form intra-and intermolecular disulfide bonds characteristic of TGFβ family proteins (Padgett et al., 1997; Massagué, 1998). Eight cysteines in the TGFβ2 form four intrachain disulfide bonds. The dimer is stabilized by the ninth cysteine, which forms an interchain disulfide bond, and two identical hydrophobic interfaces (Daopin et al., 1992; Schlunegger and Grütter, 1992).

TGFβ is secreted as a biologically latent dimeric complex that contains the C-terminal mature domain and N-terminal pro-domain, LAP (latency associated peptide). The dimer is formed due to disulfide bond between two polypeptides of pro-domain. TGFβ is cleaved from its propeptide by furin-like endoproteinase during secretion (Koli et al., 2001; Kusakabe et al., 2008). Studies in cell cultures show the insensitivity of TGFβ2 to furin which is due to the tertiary structure of their LAP regions rather than their cleavage site (Kusakabe et al., 2008). The LAP propeptide remains associated with TGFβ dimer by non-covalent interactions. This complex is referred to as small latent TGFβ. The large latent TGFβ complex contains one of the four latent TGFβ binding proteins (LTBP) that is covalently linked to LAP (Fig.1.5). In most studied cell lines, it is found that TGFβs are secreted as large latent complexes (reviewed in Koli et al., 2001).

LTBPs that belong to the LTBP/fibrillin-family of extracellular matrix (ECM) proteins are considered to have a central role in the processing, secretion and activation of TGFβs. There are four different LTBPs cloned (Koli et al., 2001). The structures of these glycoproteins are highly repetitive, mainly composed of epidermal growth factor (EGF)-like repeats, eight cysteine (8-Cys) repeats that are unique to this family, and also flanking regions containing proline-rich areas.

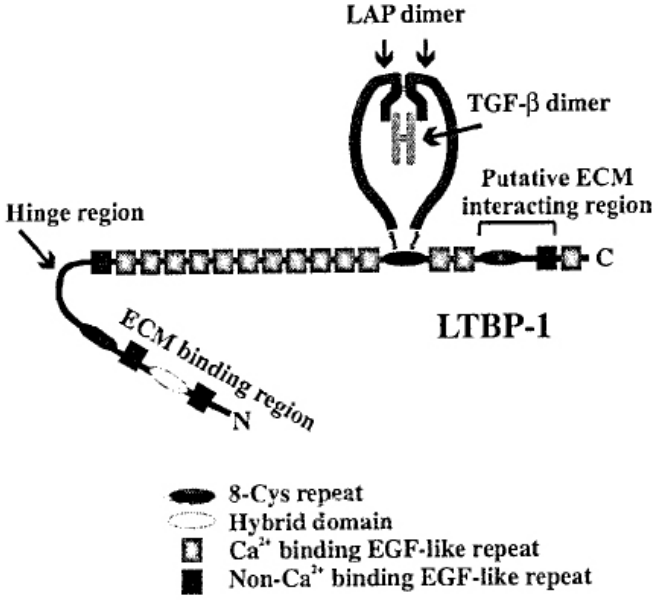

Figure. 1.5. Schematic structure of the large latent TGFβ complex (Koli et al., 2001). The small latent TGFβ complex contains the mature protein on the C-terminal, and LAP (latency associated peptide) in its N-terminal. This structure is linked via disulfide bond to the third 8-Cys repeat of LTBP1 (Latent TGFβ binding protein 1).

LTBPs are important for folding and secretion of TGFβ and they play a role in the localization of these complexes to the ECM and in the activation processes of TGFβs (Taipale et al., 1994). Additionally, LTBP1 has functions as a chemo-attractant (Kanzaki et al., 1998) whereas LTBP2 has been reported as an anti-adhesive ECM protein (Koli et al., 2001). The existence of four different LTBPs with only partial overlapping expression patterns suggests important functions in different tissues. LTBP1 is mainly expressed in the heart, placenta, lung, spleen, kidney and stomach whereas LTBP2 is expressed in the lung, skeletal muscle, liver and placenta. LTBP3 and LTBP4 have similar expression patterns and are found in aorta, heart, small intestine and ovaries (Koli et al., 2001). Interestingly, in fetal tissues, LTBP1 and LTBP2 have higher expression than LTBP3 and LTBP4 (Saharinen et al., 1998).

The central parts of all LTBPs are composed of a long stretch of 9 - 14 EGF-like repeats (Fig.1.5); this region is resistant to proteolysis (Taipale et al., 1994; Saharinen et al., 1998). These repeats participate in protein-protein interactions and also provide stability to protein structures via calcium binding (Koli et al., 2001). LTBPs mediate binding of all three TGFβ isoforms to fibroblast-like ECM matrices (Olofsson et al., 1992). The N-terminal parts and also parts of C-terminus of LTBP are important in ECM/TGFβ association. The N-terminus contains transglutaminase substrate motifs. Transglutaminase is required for covalent ECM association (Fig. 1.6; Nunes et al., 1997).

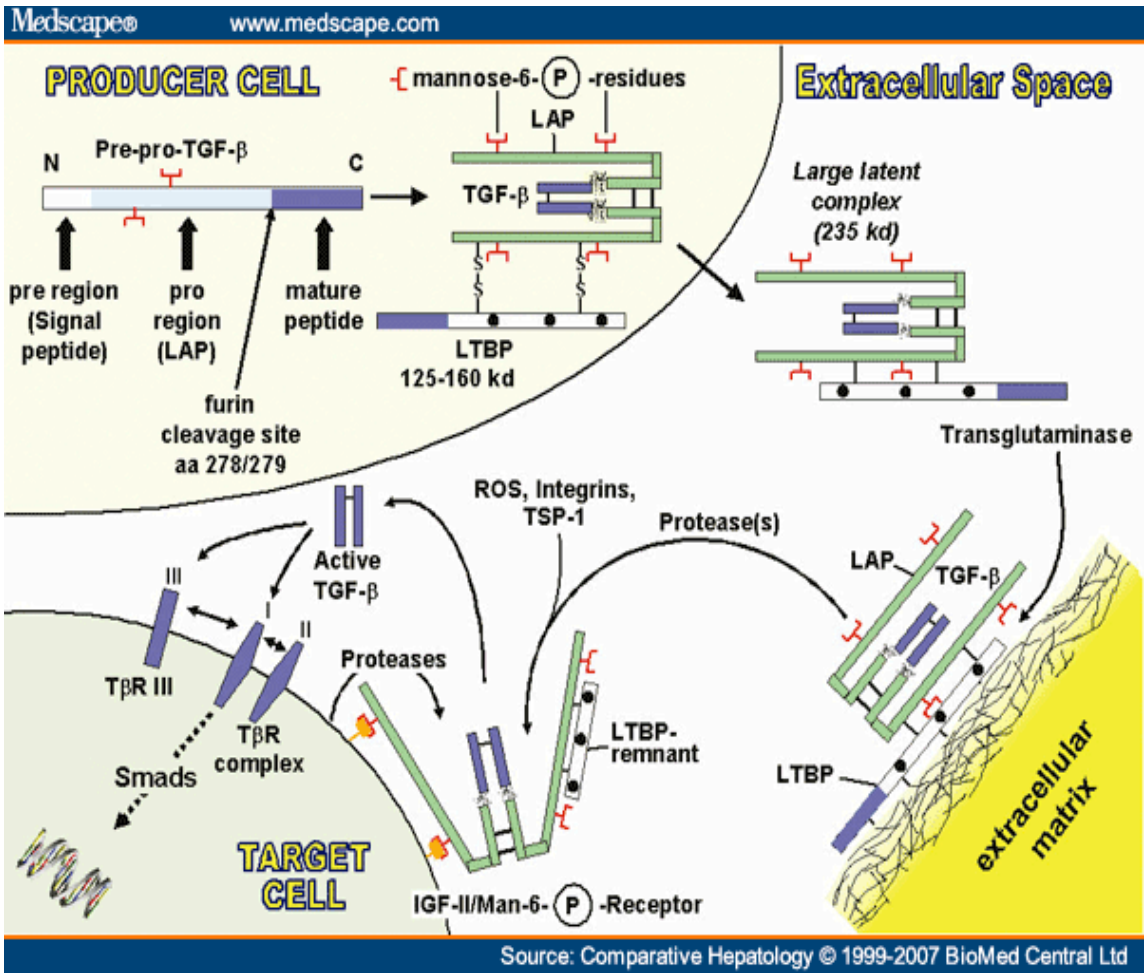

Figure 1.6. Extracellular matrix and TGF-β (Gressner et al., 2007). Schematic presentation of intracellular TGF-β synthesis, secretion and extracellular immobilization via transglutaminase-dependent fixation of the large latent TGF-β binding protein (LTBP) to extracellular matrix, release by proteases and activation of the latent TGF-β complex by reactive oxygen species (ROS), specific integrins, thrombospondin-1 (TSP-1) or proteases with release of the active TGF-β homodimer, which binds to TGF-β receptors (TGFβR) III, II, and I to initiate the intracellular signaling cascade of Smad phosphorylation. Regulation of TGF-β occurs at the transcriptional level and, most importantly, by extracellular activation. LAP - latency associated peptide.

LTBPs are produced in excess of TGFβ the secretion of which is inefficient in the absence of LTBP (Miyazono et al., 1991). Release of active TGFβ from the complex that is associated to the ECM, requires two steps, i.e. the release of complex from ECM by proteolysis and an activation of TGFβ which can be achieved by different mechanisms. TGFβ regulates the cellular production of ECM components as well as the proteolytic balance thus maintaining the organization of extracellular structures (Koli et al., 2001).The major fraction of secreted LTBPs does not contain TGFβ (Miyazono et al., 1991). Cancer cells have often been found to produce abnormal amounts of TGFβ; they also fail to deposit TGFβ complexes to the ECM, probably due to decreased fibronectin matrix in cancer cells and a decreased production of LTBP (Mizoi et al., 1993; Taipale et al., 1998).

The proteolytic release of TGFβ from ECM is driven by multiple proteinases of the serine protease family, including plasmin, mast cell chymase and leukocyte elastase (Taipale et al., 1995).

The released TGFβ is still in a biologically inactive form protected by the domain consisting of EGF-like repeats forming well protected structure that can be activated by acidification or heat treatment (Taipale et al., 1994). Release from the ECM involves the cleavage of the LTBP1 and LTBP2 at protease-sensitive sites (hinge region) whereas LTBP3 and LTBP4 are not cleaved at this region (Fig.1.6; Rifkin, 2004). Plasmin cleaves also LTBP2 thus releasing a large soluble protease-resistant fragment (Hyytiäinen et al., 1998).

Activation of latent TGFβ involves the disruption of the non-covalent interaction between the LAP and TGFβ, thus enabling TGFβ to bind its signaling receptors localized within the cellular surface. Two major mechanisms of activation of latent TGFβs are described in the literature; dissociation from LAP activates the TGFβs superfamily ligands and conformational changes such that the LAP is not released from its association with TGFβ complex but exposes the TGFβ receptor binding site (Khali, 1999; Rifkin, 2004).

The existence of different TGFβ isoforms, latent complexes, and LTBPs, suggests that there are multiple pathways for the activation of TGFβ. TGFβ can be activated *in vitro* by multiple physicochemical, enzymatic and drug-induced mechanisms (Koli et al., 2001). Physiochemical activation includes acidification of cellular microenvironment, changes in pH, irradiation and reactive oxygen species. From a physiological point of view, the acidic environment during wound healing could induce activation of TGFβ (Koli et al., 2001).

Proteolysis targets the degradation of LAP pro-peptide and thus, the releases of active TGFβ. Mechanisms involving proteolysis are more diverse and more likely to operate *in vivo*. The proteolytic release of large, latent TGFβ from ECM may regulate local TGFβ activity either positively on negatively, depending on whether the substrate for activation is the soluble or matrix-bound form of latent TGFβ (respectively) (Koli et al., 2001). Also, LTBPs take a part in proteolysis mediated TGFβ activation, since excess free LTBP or specific LTBP antibodies inhibit the activation process (Koli et al., 2001). Plasmin-mediated TGFβ activation can be neutralized through feedback inhibition, because TGFβ induces production of plasminogen activator inhibitor-1 (PAI-1) thus decreasing the formation of active plasmin (Laiho et al., 1986). Another ECM protein, thrombospondin, a platelet α-granule induced during wound healing, has been proposed to be involved in activation of TGFβ complexes via mechanisms that do not involve cell surface proteases but induce changes in the conformation of LAP thus releasing active TGFβ (Ribeiro et al., 1999).

Steroid hormone superfamily members are efficient regulators of the expression of TGFβ isoforms. TGFβs are likely to act as local mediators of the diverse actions of steroids, e.g. estrogens

and anti-estrogens can regulate TGFβ1 formation in different cells and tissues (Sporn and Roberts, 1992). There have been reports of retinoid induced TGFβ activity (Glick et al, 1989; 1991). Retinoic acid and high calcium induce the expression of TGFβ2 in epidermal keratinocytes. Using neutralizing antibodies against TGFβ2, it has been shown that TGF β2 mediates the growth-inhibitory effects of retinoic acid (Glick et al., 1991). However, recent reports show opposite retinoic-acid response of TGFβ2 in developing heart in early embryogenesis of mouse (Li et al., 2010) and chicken embryo (Romano and Runyan, 2000; Mercado-Pimentel and Runyan, 2007). High concentrations of vitamin D and its derivatives can induce the expression of the TGFβ in keratinocytes. Activity measurements indicate that a fraction of induced TGFβ is biologically active without heat or acid treatments, but only 15% of the total, acid-activatable TGFβ was found to be active (Koli and Keski-Oja, 1993).

The TGFβ superfamily ligands signal through their receptors that belong to the family of transmembrane serine/threonine kinases. Based on their structural and functional properties, they are divided into three classes: TGFβRI, TGFβRII and TGFβRIII which include a ubiquitous extracellular β-glycan and the membrane glycoprotein endoglin (CD105) (Cheifetz et al., 1992; Kingsley, 1994). Beta-glycan binds to TGFβ2 with a greater affinity than other TGFβ isoforms and facilitates it interactions with TGFβRII/I complex, since TGFβ2 has lower affinity for TGFβRII than TGFβ1 and TGFβ3 (López-Casillas et al., 1991; Wang et al., 1991). In contrast, endoglin interacts with TGFβ1 and TGFβ3 isoforms, but has low affinity for TGFβ2 (Cheifetz et al., 1992; Vincent et al., 1998). Beta-glycan belongs to the class of cell surface receptor molecules that regulate the access of ligands to the signaling receptors and contains heparan and chondroitin sulfate chains whose core protein binds to TGFβs (Cheifetz et al., 1992). Membrane-bound beta-glycan positively regulates TGFβ2 whereas a soluble form of beta-glycan found in serum and ECM (Andres et al., 1989) is capable of binding TGFβ2 with high affinity thus inhibiting TGFβ2 signaling (López-Casillas et al., 1994). Because of this dual action it has been suggested that beta-glycan can be one of the TGFβ regulators *in vivo* (Esparza-López et al., 2001). However there is evidence of TGFβ2 signaling in absence of TGFβRIII. A splice variant of the TGFβRII allows TGFβ2 ligand-receptor interaction in the absence of the TGFβRIII (Rotzer et al., 2001) as well as TGFβRII requires TGFβRI in order to bind the TGFβ2 (del Re et al., 2004).

In mammals, only five type II receptors and seven type I receptors have been identified, whereas 29 ligands have been found (Heldin et al., 1997; Massagué, 1998; Derynck and Zhang, 2003). TGFβ type I and type II receptors are glycoproteins and interact upon ligand binding. While TGFβRIII modulates ligand access to the signaling receptors, TGFβRI and TGFβRII are responsible

for signal transduction. Evidence from several studies led to the heterodimeric receptor model which postulates that TGFβRI requires TGFβRII for ligand binding and TGFβRI and the kinase activity of TGFβRII are required for signaling (Wrana et al., 1994). TGFβ1, TGFβ3 and activins bind type II receptor without requiring type I receptor, while BMP2, BMP4 and BMP7 bind primarily to their type I receptors (Feng and Derynck, 2005). TGFβ2 interacts only with the type II/ type I receptor combinations. Ligand binding to homomeric receptor dimers is not sufficient to activate signaling (Derynck and Zhang, 2003). Activation of TGFβRI and subsequent signaling requires phosphorylation of its Gly-Ser (GS) domain by TGFβRII in the heterodimeric complex. Ligand binding to the type II receptor can induce cytoplasmic domain autophosphorylation; however type II receptor signaling in the absence of type I receptors has not been reported (Derynck and Zhang, 2003). Phosphorylation provides the basis of downstream signaling (Wrana et al., 1994; Böttner et al., 2000).

1.2..2. SMAD dependent TGFβ signaling

TGFβ/activin and BMP typically use different signal transduction pathways. TGFβ and activin must bind type II receptor that will phosphorylate type I receptor that will phosphorylate SMAD2 and SMAD3, allowing each to migrate to the nucleus, interact with SMAD4, and transcription factors or co-regulators, and stimulate TGFβ target genes. BMPs will bind to type I or type II receptor; signal transduction will be directed through SMAD1, SMAD5, or SMAD8 that will also interact with SMAD4 in the nucleus and regulate BMP target genes. SMAD proteins are intracellular components of the TGFβ signal transduction pathway. The first member of this family is MAD (mothers against decapentaplegic) which was identified in *Drosophila melanogaster* (Sekelsky et al., 1995). Other members of this family were identified on the basis of their sequence homology with MAD. Homologs in *Caenorhabditis elegans* have been named *sma* because the mutations in these genes resulted in smaller body size of larva. Vertebrate homologs of sma and MAD are called SMAD (Derynck et al., 1996). At least 10 vertebrate proteins have been identified. Members of this family play different roles in TGFβ superfamily signaling. SMAD1, 2, 3, 5 and 8 can be phosphorylated by TGFβRI after ligand-induced dimerization of TGFβRI and TGFβRII; therefore they are called receptor-regulated SMADS (R-SMAD) (Fig.1.7) (Massagué, 1998; Massagué and Chen, 2000). The phosphorylation of these SMADS leads to their localization from the cytoplasm to the nucleus, and enables them to interact with common SMAD4 (co-SMAD) that

form hetero-oligomers with R-SMADs and translocate into the nucleus upon the activation of signaling pathways (Fig.1.7, Massagué and Chen, 2000).

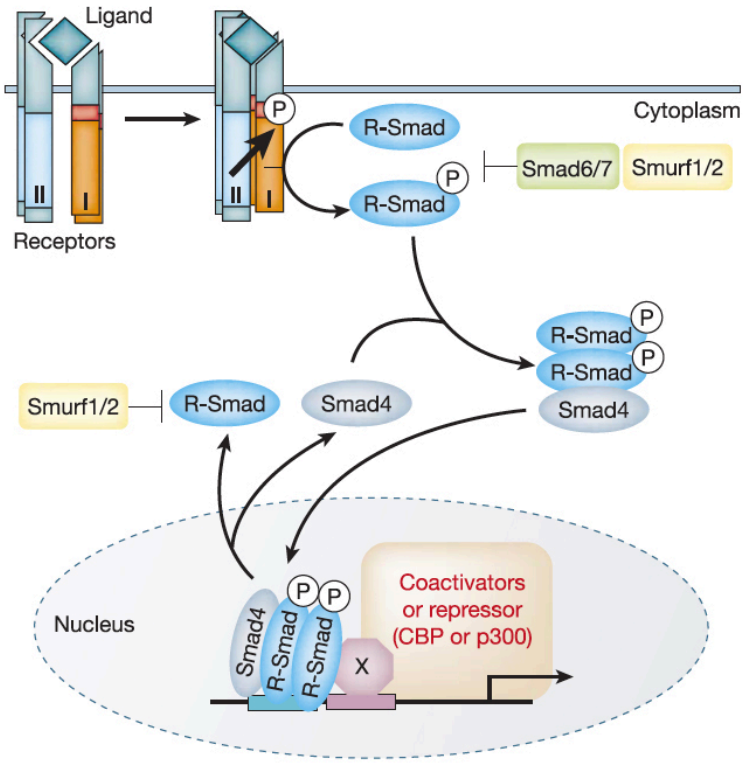

Figure 1.7. Schematic mechanism of TGFβ signal transduction through receptor and SMAD activation (Derynck and Zhang, 2003). Ligand binds to its receptors on cell surface, inducing the phosphorylation of receptor SMADs (R-SMAD) that leads to oligomerization with common-SMAD (SMAD4) and translocation to the nucleus and binding to the promoter of target genes regulating its expression through physical interactions with other DNA binding transcription factors (X) and con-activators. Activation of R-SMADS is inhibited by SMAD6 or SMAD7. The R-SMADs and SMAD4 shuttle between nucleus and cytoplasm. The E3 ubiquitin ligases Smurf1 and Smurf2 mediate the degradation of R-SMADs.

Interestingly, SMAD4 is not essential for TGFβ signaling as some TGFβ responses occur in the absence of SMAD4 and SMAD4 deficient cell lines have a limited responsiveness to TGFβ (Sirard et al., 2000). The R-SMADs and the co-SMAD consist of conserved amino- and carboxy-terminal domains that form globular structures. Between these domains lies a linker region that interacts with prolyl-isomerases and ubiquitin ligases and that is enriched in prolines and phosphorylatable serines or threonines. With exception of SMAD2, the amino-terminal Mad-homology 1 (MH1) domains can interact with other proteins and carries nuclear localization signals and a DNA-binding domain. The MH2 domain in C-terminus of R-SMADs can bind to type I receptors and can interact with other proteins, and it also mediates SMAD homo and hetero-oligomerization and mediates the transactivation potential of nuclear SMAD complexes receptor directed phosphorylation relieve these two domains (MH1 and MH2) from a mutually inhibitory interaction (Massagué and Chen,

2000; Derynck and Zhang, 2003; Moustakas and Heldin, 2009). R-SMADs and SMAD4 are expressed in most, if not all cell types; however they are differentially controlled during development. Since the C-terminal phosphorylation of R-SMADs allow them to associate with the co-SMAD, SMAD4, the resulting SMAD complex consist of a trimer of two R-SMADs and a single SMAD4 (e.g. SMAD2-SMAD-2-SMAD4 complex, a SMAD3-SMAD3-SMAD4 complex, or a SMAD2-SMAD3-SMAD4 complex), which then translocates to the nucleus where it bind to chromatin and regulate gene expression together with other transcription factors (Massagué et al., 2005). The SMAD-binding element (SBE) is defined as the sequence 5'-AGAC-3'or its reverse complement 5'-GTCT-3' (Derynck and Zhang, 2003).

The third class of SMADs includes inhibitory SMAD6 and SMAD7 and the only known function of them is to inhibit the signaling activity of R-SMADs. Inhibitory SMADs lack recognizable MH1 domain, but have MH2 domain (Derynck and Zhang, 2003). SMAD6 can inhibit BMP signaling, whereas SMAD7 inhibits both TGFβ and BMP signaling by occupying the TGFβRI for BMPs and TGFβs. SMAD6 inhibits signaling by competing with co-SMAD for binding to receptor-activated SMAD1 and forms inactive SMAD1/SMAD6 complex (Massagué, 1998). SMAD6 and SMAD7 levels are elevated in response to BMP and TGFβ signaling thus these SMADs function as negative feedback controls for different pathways including TGFβ (Chang et al., 2000; Derynck and Zhang, 2003). Activation of the epidermal growth factor (EGF) receptor and possibly other tyrosine kinase receptors, interferon-γ signaling through STAT (signal transducer and activator of transcription) proteins, and activation of NF-κB by tumor necrosis factor α (TNFα), also induce SMAD7 expression, leading to inhibition of TGFβ signaling (Derynck and Zhang, 2003). The Smurf1 (SMAD-ubiquitination-regulatory factor 1) and Smurf2, the HECT (homologous to the E6-AP carboxy terminus) family E3 ubiquitin ligases, also antagonize TGFβ signaling by interacting with R-SMADs and targeting them to degradation. Smurf1 interacts more with SMAD1 and SMAD5 which are BMP signal transducers, whereas Smurf2 interacts more broadly with different R-SMADs, thus allowing interference with BMP and TGFβ signaling (reviewed in Derynck and Zhang, 2003).

After ligand binding, the TGFβ receptors remain active for at least 3-4 h and continuous receptor activation maintains the SMAD complexes in the nucleus where they regulate gene expression (Inman et al., 2002). Without ligand stimulation R-SMADS are localized in the cytoplasm, whereas SMAD4 is distributed in the nucleus and cytoplasm. In the nucleus, R-SMADs are constantly dephosphorylated, resulting in disassociation of SMAD complexes and export inactive SMADs to the cytoplasm (Inman et al., 2002). In opposite to ligand dependent import of R-

SMADs, SMAD4 continuously shuttles between nucleus and cytoplasm because of combined activities of a constitutively active nuclear localization sequences in the MH1 domain and a nuclear export signal in the linker region whose activity depends on the nuclear transport receptor CRMI (Inman et al., 2002; Derynck and Zhang, 2003).

SMAD phosphorylation of C-terminus of the type I receptor is the crucial point in SMAD-dependent signaling, but other kinase pathways can further regulate SMAD signaling (Derynck and Zhang, 2003). The Erk mitogen-activated protein kinase (MAPK) pathway, stimulated by the activation of tyrosine kinase receptors and Ras can target R-SMADs. Erk MAPK phosphorylates the MH1 domain of SMAD2 and linker segments of SMAD1, SMAD2 and SMAD3 (Funaba et al., 2002). Tyrosine kinase receptor activation and oncogenic Ras inhibit ligand-induced nuclear translocation of activated SMADs (Funaba et al., 2002). Phosphorylation of SMADs can also occur from the activation of MAPK/Erk kinase kinase 1 (MEKK1), which acts downstream from Ras and upstream from growth factor induced Erk MAPK and stress-activated SAP/JNK (c-Jun N-terminal kinase) pathways. MEKK1 activation enhances SMAD2 phosphorylation, heterodimerization with SMAD4, nuclear translocation and transcriptional activity (reviewed in Derynck and Zhang, 2003). The induction of Erk MAPK and JNK signaling by TGFβ itself may regulate SMAD activation and signaling (Derynck and Zhang, 2003).

Since the specificity between ligands and their receptors is only partial, the functional specificity of TGFβs probably depends upon extracellular ligand activation, differences in intracellular SMAD interactions, interactions with other signaling pathways like Ras/MAPK, and interactions between SMADs and transcriptional co-activators and co-repressors (Ahzar, 2003).

1.2.3. SMAD-independent TGFβ signal transduction

Other proteins mediate TGFβ signaling in addition to SMADs. In a breast epithelial cell culture model it has been demonstrated that TGFβ activated TGFβRII phosphorylates the polarity protein PAR6, which regulates the local degradation of Rho, a small GTPase that controls assembly of intercellular tight junctions in mammalian cells and as these junctions dissemble, epithelial architecture disintegrates followed by EMT (epithelial-mesenchymal transition) that is regulated by TGFβ signaling (Moustakas and Heldin, 2009). While in the breast epithelial cell culture model TGFβ/PAR6 pathway locally degrades Rho, other studies demonstrate positive activation of Rho GTPase signaling by TGFβ and BMP receptors in different cell types (Kardassis et al., 2009).

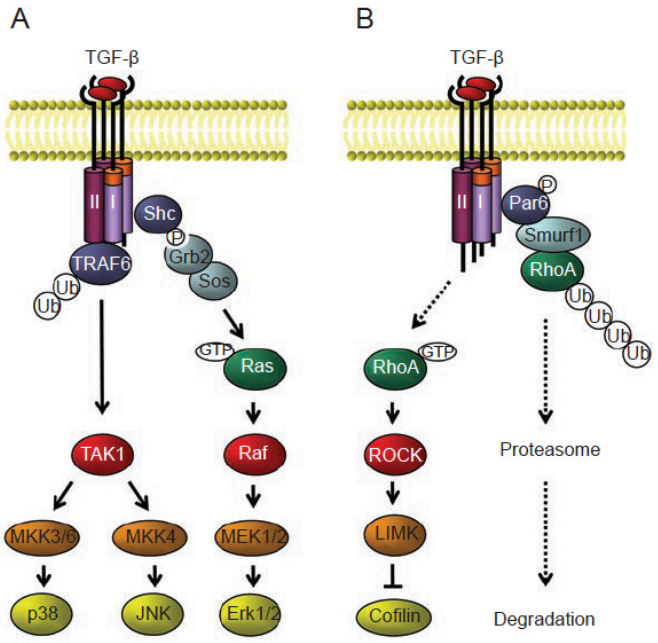

Figure 1.8. Schematic example of SMAD-independent TGFβ signaling (Xu et al., 2009). A, TGF-β activates p38 MAP K and JNK/MAPK signaling through the activation of TAK1 by receptor-associated TRAF6, and Erk MAP kinase signaling through recruitment and phosphorylation of Shc by the TβRI receptor. B**,** activation of RhoA in response to TGF-β and induction of ubiquitin-mediated RhoA degradation at tight junctions.

Small GTPases control the early TGFβ signaling towards actin cytoskeleton reorganization via non-SMAD pathways, whereas late cytoskeletal events are suggested to be directed by specific cross-talk between SMAD-mediated transcriptional events involving up-regulation of Rho proteins or cytoskeletal proteins (Kardassis et al., 2009). This regulation is biologically significant during the EMT process not only in embryonic development, but also in cancer cells.

The TGFβRI directly phosphorylates both serine and tyrosine residues in the ShcA adaptor protein thus inducing its association with adaptor protein (Grb2) and the Ras guanine exchange factor son of sevenless (Sos), that leads to activation of the Ras-Raf-MEK-Erk mitogen activated protein kinase (MAPK) signaling cascade, which can regulate cell proliferation and migration (Fig. 1.8) (Lee et al., 2007). In addition, tyrosine kinase Src can phosphorylate Tyr2854 in the cytoplasmic domain of TGFβRII, leading to Grb2 and Shc recruitment and to activation of the p38 MAPK pathway that promotes oncogenic signaling by TGFβ in malignant mammary epithelial cells (Galliher and Schiemann, 2007). Another example of non-SMAD signal transduction lies within the ubiquitin ligase tumor necrosis factor α receptor-associated factor 6 (TRAF6) recruitment to the TGFβRI cytoplasmic domain in mammalian cells. TRAF6 ubiquitylates and activates the catalytic activity of the TGFβ-activated kinase 1 (TAK1; MAP3K7), leading to activation of the p38 and c-Jun N-terminal kinase (JNK) cascades, which regulate apoptosis or cell migration (Sorrentino et al., 2008). The TGFβRI kinase activity is dispensable for this pathway (Sorrentino et al., 2008). The

interplay between SMAD and JNK or p38 pathways could underlie diverse forms of integration and reciprocal regulation between TGFβ signaling and other pathways.

1.2.4. TGFβ and other pathway cross-talk

Besides SMAD-mediated transcription, TGFβ activates other signaling cascades, including MAPK pathways. MAPKs, including Erk1/2, JNK1/2/3 and p38MAPKs are evolutionary conserved regulators that are essential for various cellular events. Many extracellular stimuli can induce the activation of serial phosphorylation from MAP kinase kinase kinase (MAPKKK) to MAP kinase kinase (MAPKK) and finally to MAPK which then phosphorylates many proteins (mostly nuclear transcription factors) with diverse functions in regulating proliferation, cell growth, migration (Chang and Karin, 2001). One of the best characterized triggers for MAPK pathway is Ras activation which can propagate signals from various ligand or self-activated tyrosine kinases. A general notion from several studies have shown that HER2/Ras can antagonize TGFβ induced cell apoptosis and cell cycle arrest while allowing for the pro-migratory and pro-invasive functions of TGFβ (Seton-Rogers et al., 2004). The synergy between the TGFβ and HER2/Ras/MAPK pathways often leads to the secretion of additional growth factors and cytokines, including TGFβ itself, which promotes epithelial-mesenchymal transition (EMT) and cell invasion, whereas JNK kinases negatively regulate the autocrine expression of TGFβ (Ventura et al., 2004).

The SMAD linker region is loosely organized and highly flexible in structure that makes it accessible for a number of kinases. Three residues in the linker region of SMAD3 were identified as Erk1/2 phosphorylation sites both *in vivo* and *in vitro*. Erk-mediated phosphorylation in these sites inhibits SMAD3 transcriptional activity (Matsuura et al., 2005). MAPKs also phosphorylate the linker region in SMAD1/5 thus blocking SMAD1/5 nuclear translocation, resulting in inhibition of BMP signaling by several signals that activate MAPK (e.g. EGF, FGF, IGF) (Guo and Wang, 2009). Erk, JNK, and p38 have all been implicated in the transcriptional regulation of SMAD7, whereas JNK and p38 phosphorylate tumor-derived SMAD4 and promote its degradation, thus indirectly regulating TGFβ signaling (Guo and Wang, 2009).

MAPK phosphorylate number of nuclear transcription factors, including those that can interact with SMADs and regulate TGFβ/BMP responses. Best characterized are AP1 proteins including members of Jun, Fos, Maf and ATF subfamilies (Shaulian and Karin, 2002; Guo and Wang, 2009). Relationship between Jun/Fos and SMAD functional interactions can be synergistic or antagonistic depending on their target genes and other binding proteins.

TGFβ and Wnt family members regulate various developmental processes; several differentiation and patterning processes require inputs from both pathways. In vertebrates, signaling of both pathways is important for establishing the basic body plan during gastrulation. It has been shown that mice lacking Wnt central signal transduction molecule, β-catenin, fail to form anterior-posterior axis (Huelsken et al., 2000); the same effect is observed in mice lacking TGFβ superfamily signaling molecules, like SMAD2 and SMAD4 (Weinstein et al., 2000). Several studies in *Drosophyla* and *Xenopus* have shown that TGFβs and Wnt cooperate in regulating developmental and patterning events through a coordinate control of gene expression patterns (Attisano and Labbé, 2004). In vertebrates, Wnts have been shown to cooperate with BMP-like ligands, by regulating graded Emx2 expression in dorsal telencephalon (Theil et al., 2002) and in embryonic carcinoma cells, Wnt and BMP have cooperative effect in several gene targets including Msx1 and Msx2 (Willert et al., 2002). Studies with mouse models have revealed the cooperative involvement of TGFβ and Wnt signaling in cancer where disruption of TGFβ signaling in the context of activated Wnt signaling, promotes tumorigenesis (Cullingworth et al., 2002).

The requirement of both pathways has been explained through an indirect effect as shown in studies with chicken embryo where mis-expression of Vg1, a TGFβ-like ligand, causes induction of Wnt signaling pathway transcription factor LEF-1 expression (Skromne and Stern, 2001). Analysis of specific target genes has revealed that members of both families can regulate gene expression through distinct but closely positioned enhancer elements, suggesting that the synergism between TGFβ and Wnt might occur more directly (Attisano and Labbé, 2004). In studies with *Xenopus*, it has been shown that maximal activation of Xtwn gene requires presence of four components, R-SMAD, SMAD4, LEF-1 and β-catenin suggesting that TGFβ and Wnt pathways synergistically activate specific target genes through direct interaction of SMADs and LEF-1 and this synergy can activate a specific gene expression program and thereby direct appropriate tissue patterning (Attisano and Labbé, 2004).

Interactions of TGFβ/BMP pathway with other pathways such as PI3K/Akt, Sonic Hedeghog (Shh), Notch, IL, TNFβ and INF-γ pathways have been described (reviewed in Guo and Wang, 2009). The core concept of TGFβ signaling cross-talk is the context dependency leading to conclusion that no simple role can be generalized to describe how TGFβ interacts with any other signaling pathway.

1.2.5. TGFβ in vertebrate embryonic development

Mouse models with targeted disruption of TGFβ gene expression reveal the role of each isoform during mouse development. TGFβ1 disruption results in diffuse and lethal inflammation in about three weeks after birth; also embryonal hematopoiesis is also defective (Shull et al., 1992; Kulkarni et al., 1993; Dickson et al., 1995; Letterio and Roberts, 1996). Alterations in both TGFβ2 and TGFβ3 genes lead to perinatal lethality. TGFβ2 null mice exhibit cardiac, lung, craniofacial, limb, eye, ear and urogenital developmental defects (Sanford et al., 1997) while TGFβ3 null mice have defective palatogenesis and arrested pulmonary development (Kaartinen et al., 1995; Proetzel et al., 1995).

During vertebrate embryogenesis, the heart is the first organ to form and function. The heart initially forms from two bilateral heart fields that fuse at the ventral midline to form the primitive heart tube consisting of an inner endothelium surrounded by an outer layer of myocardium. Between these layers is extracellular matrix layer called cardiac jelly secreted mainly by the myocardium. Subsequently loops and turns break the symmetry of the linear heart tube. In addition, endocardial cells undergo epithelial-mesenchymal transition (EMT) in response to signals from the overlying myocardium. As a result of this transition cells invade intervening ECM and form cardiac cushion. The mature heart valves and septa derived from the cardiac cushions ultimately divide the heart into four functional chambers (Harvey, 2002).

Molecular studies have shown that TGFβ superfamily signaling is essential for heart development. For example, BMP2 is required for initial formation of the cardiac primordium, because Bmp2 null mice either do not have a heart or develop very retarded and malformed heart (Zhang and Bradley, 1996). In addition, several TGFβ isoforms and receptors control EMT in the developing heart. In the chicken, TGFβ2 and TGFβRIII have roles in cell activation suggesting a direct and specific interaction. TGFβ3 and TGFβRII appear to have similar roles in cell invasion. In the mouse, TGFβ2 is the only ligand that has a demonstrated role in EMT. Fusion of the cardiac cushions fails in TGFβ2 knock-out mice, due to an increased number of mesenchymal cells that do not form cell-cell contacts to remodel the valves (Mercado-Pimentel and Runyan, 2007). TGFβ1 knock-out mice do not seem to have any heart abnormalities, while TGFβ2 knock-out mice have specific defects in the development in valves and septa of the heart (Sanford et al., 1997). Recent studies with mice embryos demonstrate a role for TGFβ2 in early heart morphogenesis in the formation of the outflow tracts (Li et al., 2010). Vitamin A-deficient quail embryos develop severe heart abnormalities that lead to embryonic death at day 3.5-4. Some of these abnormalities manifest

as abnormal, un-looped, randomly oriented (left/right) but beating heart, sparse and abnormal extra-embryonal blood vessels and the absence of linkages between the heart and extra-embryonal blood supply; the inflow tracts are closed in the VAD embryos (Zile, 2004; 2010).

Two type I receptors, Alk3 and Alk5, also have been shown to be involved in heart development. Alk3, also known as BMP type IA receptor, is essential for mesoderm formation as shown in conventional knock-out mice (Gaussin et al., 2002). It is interesting that TGFβ2 is downstream of Alk3 during cardiac cushion formation, as suggested from findings that in Alk3 knock-out mice the levels of TGFβ2 expression are greatly reduced when Alk3 is absent from the myocardium (Gaussin et al., 2002). These findings suggest that a cascade of TFGβ superfamily signaling is required for normal cardiac cushion formation. Alk5, a receptor for TGFβ isoforms may be required for heart looping during mouse embryonic development (Charng et al., 1998).

In summary, heart development is regulated by both TGFβs and BMP isoforms at multiple developmental stages, including the time of formation of the heart primordia, heart looping and cushion formation, and later, at septum and valve formation. Findings from mouse knock-out models where TGFβ2 levels are decreased in absence of Alk3, suggest genetic connections among signaling pathways of different TGFβ superfamily members.

During mouse development, morphological asymmetry of the left/right axis occurs at about E8.0 (in chick it occurs at 10/11ss) when the embryonic heart tube loops toward the right. In chick, molecular determination of left/right axis is first initiated in Hensen's node and then manifested by asymmetrical gene expression. Several TGFβ family ligands, such as Nodal, lefty-1, and lefty-2 are expressed asymmetrically before or during the appearance of morphological asymmetry; they appear to be involved in normal left/right axis formation (reviewed in Chang et al., 2002). In chick, it appears that the chick Cerberus (cCer), a member of Cerberus/Dan family that first was identified as BMP antagonist, is a BMP agonist in Nodal induction in the chick left plate mesoderm. Thus BMP and cCer function synergistically to ensure the left-sided Nodal expression in the chick embryo during left/right axis development (Yu et al., 2008). TGFβ isoforms do not appear to be involved in this developmental process.

Vasculogenesis and angiogenesis are two processes leading to the formation of blood vessels. Vasculogenesis is the *de novo* formation of blood vessels from endothelial precursors in mesoderm and their subsequent organization into primary capillary plexus, whereas angiogenesis is the formation of blood vessels from pre-existing vessels through splitting and sprouting (Weinstein 1999; LaRue et al., 2003). Morphological observations in TGFβ1 knock-out mice demonstrate the role of TGFβ1 in vasculogenesis and hematopoiesis (Dickson et al., 1995). The disruption of

TGFβ1 in mice leads to midgestation lethality in half of the homozygotes and about half of heterozygotes, where the primary causes are defects in the yolk sack vasculature and the hematopoietic system (Dickson et al., 1995). Initial differentiation of mesodermal precursors occurs, but subsequent differentiation of endothelial cells into capillary like tubules is disturbed leading to vessels with decreased wall integrity. Interestingly, TGFβRII-deficient mice also develop a similar phenotype. However, recent studies where in cell cultures OP9 stroma cell co-cultures with mouse embryonic stem cells do induce vascular smooth muscle cell (VSMC) and pericyte differentiation, but TGFβ1 together with platelet-derived growth factor (PDGF) are dispensable for the induction of VSMC/pericyte genes in that system (Lindskog et al., 2008). There is no evidence of other TGFβ isoform involvement in vasculogenesis or angiogenesis.

Another important process during embryogenesis that appears almost simultaneously with the development of the cardiovascular system is the development of the nervous system. Studies carried out in the central and peripheral nervous system of mouse, rat, and chicken embryos showed the widespread expression of TGFβ2 and TGFβ3 with slight immunostaining for TGFβ1 (Böttner et al., 2000). Later studies show that TGFβ1 is also present in the central nervous system at neurulation stages as well as at the initial steps of spinal cord and brain development (Mecha et al., 2008). However, knock-out models of all three TGFβ isoforms do not show disruption in the central nervous system (Chang et al., 2002).

Targeted disruption of TGFβ2 in mouse leads to perinatal lethality due to various abnormalities, mostly related to heart defects (Sanford et al., 1997). However, various craniofacial defects, axial and appendicular skeletal defects, retinal hyperplasia and renal defects in majority of female mice were also observed (Sanford et al., 1997). Both TGFβ2 and TGFβ3 isoforms are related to cleft palate development, while BMP4 and activin βA have been shown to play important roles in tooth development (reviewed in Chang et al., 2002). BMP4 and BMP7 are involved in early eye development; however there is some evidence that TGFβ2 is also necessary for normal eye remodeling (Saika, 2006). TGFβ2 null mice also have abnormally large fontanels (spaces that occur between the skull bones before fusion), defects in bone growth in the occipital region of the skull and variable jaw bone defects (Sanford et al., 1997) which are consistent with the proposed roles of TGFβ2 in osteoblast differentiation and in osteoclast functioning (reviewed in Chang et al., 2002).

1.2.6. TGFβ and vitamin A

The functions of TGFβ and RA are known to merge in a variety of biological processes including embryogenesis, organ development and carcinogenesis (Roberts and Sporn, 1992). Interestingly, TG-interaction factor (TGIF) is a transcriptional repressor common to both TGFβ (as a co-repressor for TGFβ activated SMADs) and RA signaling pathways, by binding to the ligand-binding domain in RXRs and preventing transcription activation from the retinoid response elements in the promoter sequences (Bartholin et al., 2006). Developmental events linked to RA-regulated TGFβ signaling include the inner ear (Butts et al., 2005) and pancreas (Colakoglu et al., 2006). Inhibition of TGFβ signaling by endogenous RA is required for mouse lung development (Chen et al., 2007).

The data from most of the research in cell cultures relates to bi-directional cross-talk between RA and TGFβ signaling. Regulation of expression of cellular RA binding proteins I and II (CRABP-I, -II) and the expression of TGFβ3 were examined in mouse embryonic palate mesenchymal cells and demonstrated that all three TGFβ isoforms and RA down-regulated the expression of CRABP-I; TGFβ1 stimulated the expression of CRAPB-II and the expression of TGFβ3 within 24 h of treatment (Nugent and Greene, 1994). Furthermore, using the same model system, Nugent et al., (1995) showed the ability of TGFβ, including the endogenous forms, to modulate the expression of nuclear RARβ (Nugent et al., 1995). In contrast, RA had little or no effect on TGFβ1 mRNA, whereas TGFβ2 mRNA levels were stimulated. It was also shown that RA increased TGFβ2 and TGFβ3 protein release in the medium, protein being mostly in the latent or inactive form. The amount of active TGFβ released was increased relative to the total increase in TGFβ released, suggesting that RA treatment stimulated activation of latent TGFβ (Nugent et al., 1998). The elevated retinoid-induced TGFβ activity may be by the up-regulation of TGFβ receptors by RA (Yoshizawa et al., 1998). In contrast, findings from a vitamin A-deficient *in vivo* system clearly demonstrate that endogenous RA has a negative regulatory role on TGFβ1 and TGFβ2 (Li et al., 2010).

1.3. Early development of the cardiovascular system

Vertebrate cardiovascular system is the first physiological organ system that develops and forms during embryogenesis. Completion of development continues throughout all prenatal development. Many of the underlaying mechanisms are considered to be developmentally and molecularly conserved, but the subsequent septation of the chambers and the formation of the

outflow tract, varies among species depending upon the utilization of lungs (Fishman and Chien, 1997). Morphogenesis of the heart begins with the initial patterning of the embryo that determines the three axes of the embryo: anterior-posterior, dorsal-ventral and left-right. These axes are imprinted into the cellular program as cell populations expand to form the embryo and extra-embryonal tissues (Keller et al., 2000). Two basic processes are involved in cardiovascular development: one that drives morphogenesis (cell migration, proliferation and apoptosis), and one that is necessary for functional development (cell differentiation and determination of cardiovascular tissue). Both processes are regulated by the environment and signals within the embryo (Zile, 2004).

1.3.1. Early embryogenesis and the role of epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a concept proposed in cell biology studies of tissue morphogenesis during embryonic development (Hay, 2005). EMT is a highly conserved cellular program that allows polarized, immobile epithelial cells to convert to mobile mesenchymal cells. This process was initially recognized during several critical stages of embryonic development, but more recently it has been linked to promotion, carcinoma invasion and metastasis (Yang and Weinberg, 2008). The earliest EMT in vertebrate development appears during gastrulation, a process that forms mesoderm and endoderm tissue from the ectoderm in which the cells are organized as an epithelial sheet. Before gastrulation chick embryo is composed of two layers, i.e. epiblast and hypoblast (Fig.1.9) in *area pellucida* from which all embryonic tissues develop.

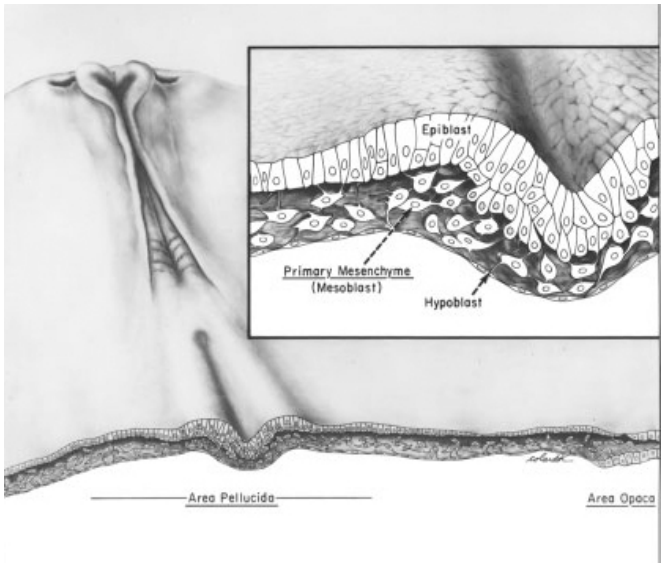

Figure 1.9. Germ layers in 24-h (4 ss) chick embryo. Somites are induced from primitive streak mesenchyme (Hay, 2005). Inset illustrates the EMT of the epiblast.

During gastrulation, the epiblast is stretched tight as a result of adhesion and cellular migration. Epiblast integrity is maintained by tight junctions between epiblast cells. Mesoderm cells form through a de-epitelialization process, i.e. EMT, from the epiblast. The primitive streak, the site of EMT, is a dynamic structure. Elongation of streak towards anterior part occurs during pre-somatic stage (HH2 to HH4) and is regulated by global tissue morphogenic movement within epiblast plane. A number of developmental factors, not related directly to EMT, influence the time and place for EMT process to generate mesoderm and endoderm (Hay, 2005; Nakaya and Sheng, 2009).

During EMT, epithelial cells undergo dramatic phenotype changes, loose expression of E-cadherin and other components of epithelial cell junction (Fig.1.10). They adopt a phenotype of mesenchymal cells and acquire mobility and invasive properties that allow them to migrate through the extracellular matrix.

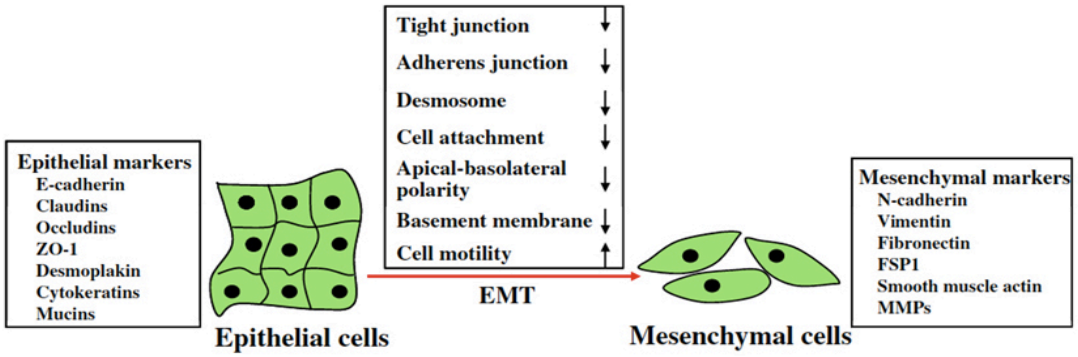

Figure 1.10. Overview of cellular changes and molecular markers during epithelial-mesenchymal transition (EMT) (Ouyang et al., 2010).

The basic types of tissue in vertebrates are epithelium and mesenchyme. There are three types of epithelium: simple, stratified and gland epithelium. Embryonic epithelium mainly is the simple type; mesenchyme develops simultaneously with epithelium (Hay, 2005). Epithelium is the earliest embryonic tissue that forms sheets of cells with tight junctions. This sheet of adjacent cells are attached tightly to the underlaying extracellular matrix consisting of collagen type IV and laminin, and is called basal lamina. Epithelial cells under tension develop very strong junctions called desmosomes which contain specialized adherence molecules (cadherins and catenins) that dissociate during EMT. These specialized cadherins are found in later stages of development, i.e. in endothelium (VE-cadherin) and in fibroblasts (N-cadherin). One of the features that separates epithelial cells from mesenchymal cells is the polarity of epithelial cells: their apical side is free and unattached, whereas the posterior side through basal actin cortex is attached to the ECM by means of integrins, the receptors for ECM.

EMT is necessary during heart development for the formation of valves, the process that occurs at 16-22 ss (HH12 to HH14) (at about 69 h of incubation) (Runyan and Markwald, 1983). The valve forming region includes atrioventricular canal and the outflow tract region that gives rise to the aortic and pulmonary valves. Prior to EMT, myocardium and endothelium are separated by a cell-free space filled with ECM, called cardiac jelly (Krug et al., 1987). In the chick, TGFβ2 and TGFβRIII have roles in cell activation that suggest direct and specific interactions, while in mice TGFβ2 is the only ligand that has a demonstrated role in EMT (Mercado-Pimentel and Runyan, 2007).

1.3.2.Early development of the heart

Heart develops from one of the three germ layers established in the early embryo. The early vertebrate embryo is an oval disk consisting of endodermal and ectodermal cell layers, between which the mesoderm is situated. Mesoderm is the germ layer that gives rise to heart muscle. The cardiovascular progenitor cells as a dense mass of mesodermal mesenchyme, move from their original midline primitive streak (under the Hensen's node) (Fig.1.11) towards forming heart fields (Pérez-Pomares et al., 2009).

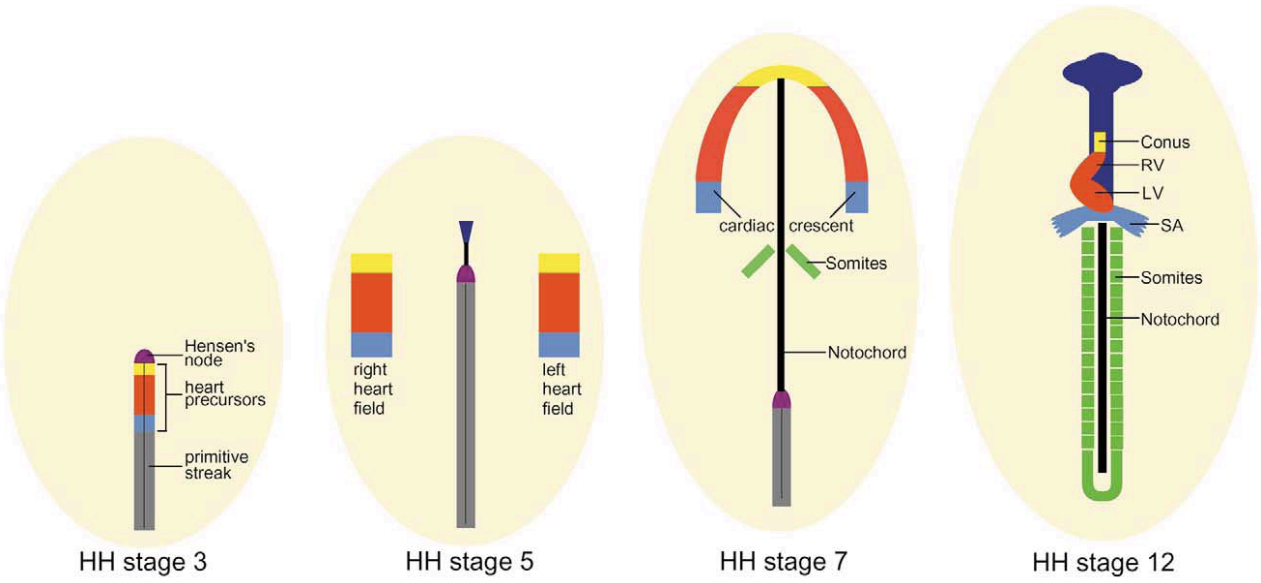

Figure 1.11. Localization of heart precursors in the primary heart field during gastrulation and tubular heart formation in avian embryo (Brand, 2003). Blue label, atrium; red label, ventricle; yellow label, conus/ outflow tract; RV, right ventricle; LV, left ventricle; SA, sinoatrial segment/inflow tract/sinus venosus.

Current theory suggests that there are two heart fields forming within anterior lateral mesoderm during early embryogenesis (Abu-Issa and Kirby, 2004, 2008; Cui et al., 2009). Primary heart field will give rise to the left ventricle and inflow tract, and the secondary heart field will

become right ventricle and the outflow tract (Black, 2007). Both heart fields are starting to form at primitive streak stage/pre-somite stage, (HH3-HH5; approximately 15h after gastrulation) in avian embryos (Fig.1.11); in the mouse it is at approximately E6-7.25 (day of embryonic development); in human it takes place at approximtely day 15 of embryogenesis. These cells will contribute to all cell layers of the heart: myocardium, endocardium and parietal pericardium, as well as to endothelial cells in the heart area (Fishman and Chien, 1997). At 1-4ss (HH7) in chicken (E7.5-E8.25 in mouse), when the lateral plate mesoderm splits into the splanchnic and somatic mesoderm, cardiovascular precursor cells and the myocardial progenitors (restricted to the splanchnic mesoderm) migrate and form the cardiac crest (horseshoe-shaped area located cranially to the prechordal plate) after which they differentiate and assemble into two bilateral heart tubes (Fig. 1.12).

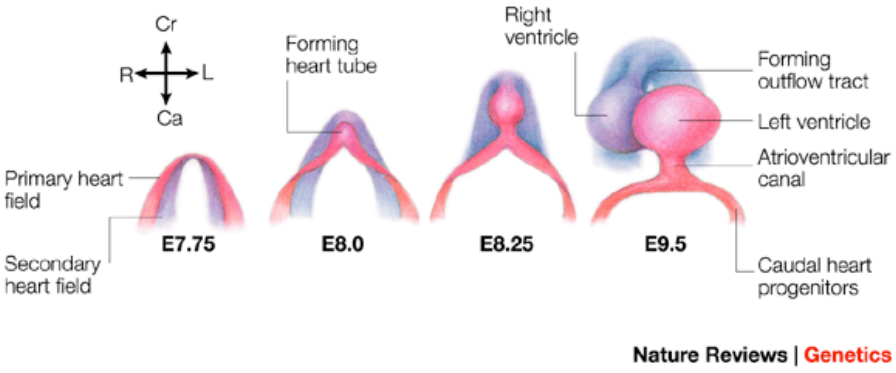

Figure 1.12. Position and movement of primary (red) and secondary (blue) heart fields from cardiac crescent through the looping stages of heart development in the mouse. Compass indicates body axes. E, approximate stages in embryonic days of development. Ca, caudal; Cr, cranial; L, left; R, right. (Harvey, 2002).

Splanchnic mesoderm (cardiogenic mesoderm) will later contribute to the most cranial portion of the heart tube (outflow tract) and the most lateral part of this mesoderm will contribute to the caudal heart tube (inflow tract) (Fig.1.13) (Abu-Issa and Kirby, 2008). The primordia of the consecutive heart chambers are definable before the appearance of myosin-type filaments and primitive Z bands, which occurs simultaneously with the first spontaneous action potentials in the 7 ss avian embryo. The bilateral heart fields move medially and fuse at the ventral midline to form the heart tube (Harvey, 2002; Abu-Issa and Kirby, 2008; Pérez-Pomares et al., 2009).

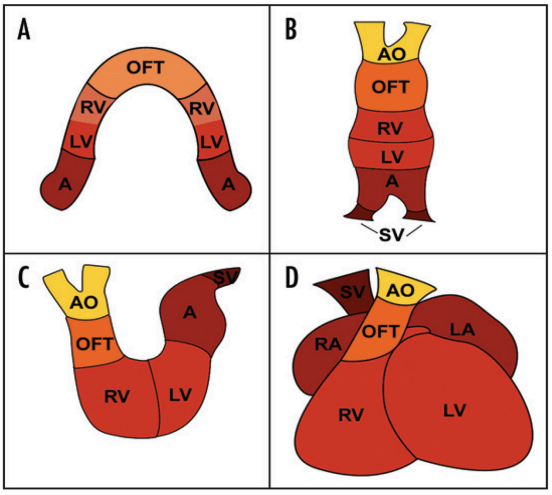

Figure 1.13. An overview of the development of the vertebrate heart. Equivalent of mouse E7.5 (A), E8.5 (B), E9.5 (C), and E11.5 (D). OFT, outflow tract; RV, right ventricle; LV, left ventricle; A, atria; AO, aorta; SV, sinus venosus (inflow tract); RA, right atrium; LA, left atrium. (Goetz and Conlon, 2007).

The bilateral heart fields initiate their differentiation into the endocardial and myocardial lineages while moving to the midline and forming the cardiac crest as the result of the forces generated by the general folding of the embryo. This process happens faster in mouse embryos than in chicken embryos (Abu-Issa et al., 2004). At the 8-9 ss in avian embryo (E8.5 in mouse), fusion of the lateral heart primordia proceeds to include the outflow tract and atrial primordia; the pacemaker site migrates into the atrial wall and subsequently into the *sinus venosus* (inflow tract) (Fig.1.13), this process occurring simultaneously with the progressive transformation of mesenchymal cells into cardiomyocytes (Virágh et al., 1989). The process of fusion and formation of a single heart tube begins towards moving anteriorly and proceeds caudally. By the 7-10 ss (HH9-10), the heart begins to move to the right (Fig.1.14).

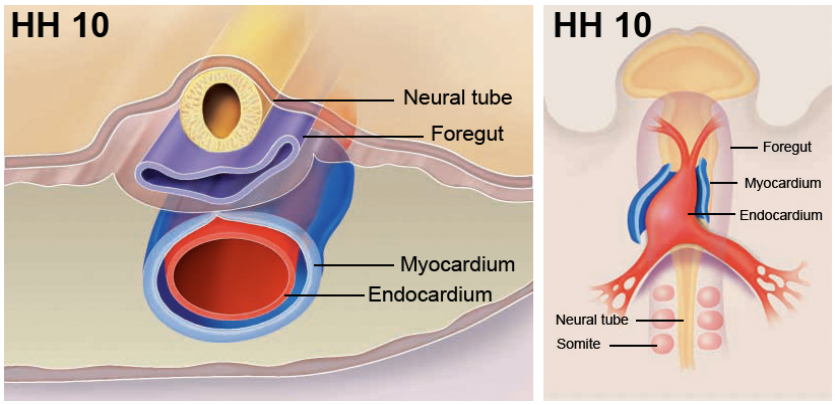

Figure 1.14. Chick heart tube at 9-10ss (HH10), shown in cross-section (left panel) and in ventral view (adapted from Fishman and Chien, 1997).

The primitive bilaminar heart tube is composed of an outer myocardial (myocardium) layer which will form the beating heart muscle, and an inner endocardial (endocardium) layer, which will form the endothelial layer of the heart contiguous with the vasculature (Fig.1.14) (Harvey, 2002; Goetz and Conlon, 2007). Endocardium has several other functions, e.g. it plays an important role in signaling to cardiomyocytes and is required for proper formation of trabecular myocardium (Wagner and Siddiqui, 2007,b). Endocardium is involved in the signaling required for transdifferentiation of myocytes into the Purkinje fiber cells that comprise much of the cardiac conduction system (Mikawa and Hurtado, 2007). At a later stage in heart development the cells from specific endocardium region will undergo EMT to form endocardial cushions (Harris and Black, 2010).

The heart tube is polarized along cranio-caudal axis with the dominant pacemaking activity located at the inflow tract, ensuring unidirectional peristaltic-like contraction and a sinusoidal electrocardiogram (Christoffels et al., 2004).

Differentiation of cells begins in upper regions of the cardiac mesoderm plate at HH7, even prior to fusion, and proceeds caudally. At 9-10 ss (HH10), the cardiac cells continue to stream together and form presumptive atrial tissue as well as the future *sinus venosus* (inflow tract) tissue which is located caudally of the heart tube. Similarly, outlet tissue (outflow tract) is recruited from the cephalic mesoderm. Thus the heart tube extends in both cranial and caudal directions (Fig.1.14) (Ciu et al., 2009). By 24-27 ss (HH15), the two chambers, atrium and ventricle, are morphologically different, and the ventricle is beginning to thicken by the growth of the wall. Cardiac jelly separates the myocardium from endocardium and becomes thicker especially in the regions where cushions and valves will form, i.e. the outflow tract and the atrio-ventricular junction (Keller et al., 2000; Wagner and Siddiqui, 2007a).

Mammalian and avian hearts are comprised of two atria and two ventricles. The formation of the atria and ventricles involves different regulatory events (Christoffels et al., 2004) which take place at a later stage of heart development and will not be discussed in this work.

1.3.3. Genes involved in early cardiovascular development

Commitment to a cardiac fate is the result of many signals from underlaying ectoderm, which include bone morphogenic proteins (BMPs), basic fibroblast growth factors (FGFs), Wnt proteins as well as several of the TGFβ superfamily cytokines (Nemer, 2008). The mechanisms that underly cardiovascular development is still not fully understood. Several transcription factors (TFs) required for normal heart development are well described and include the GATAs. GATA family of TFs consists of six proteins. GATAs 1, 2, 3 are important regulators of hematopoietic stem cells and their derivatives, while GATA 4, 5, and 6 are expressed in various mesodermal and ectodermal derived tissues (Pikkarainen et al., 2004). GATA4 is required to allow cells to respond to cardio-inductive substances; it mediates BMP signaling by combined action with the TF Nkx2.5. During in vitro cardiogenesis, GATA4 expression precedes that of Nkx2.5 and is essential for survival of cardioblasts (Grépin et al., 1997). Synergistically with SMAD proteins, the intracellular signal effectors of TGFβ/BMP signaling, GATA4 activates Nkx2.5 transcription (Benchabane et al., 2003).

The homeobox gene Nkx2.5 is one of three transcription factor genes (Nkx2.5, GATA4 and MEF2C) that are expressed in murine and chicken cardiogenic mesoderm and that encode candidate

regulators of cardiac myogenesis (Lints et al., 1993). In the chick, Nkx2.5 expression is first seen at HH5 in antero-lateral mesoderm, and along with GATA4, requires the continuous presence of BMP and FGF8 signals until myocardial differentiation becomes independent of BMP2 at 5-7 ss (HH8) (Schlange et al., 2000; Wagner and Siddiqui, 2007). However, the Nkx2.5 null mice exhibit heart abnormalities only at the stage of looping morphogenesis (Lyons et al., 1996). Studies of Nkx2.5 expression patterns in chick and mouse models support a hypothesis of Nkx2.5 role in modulating the unique patterns of gene expression involved in the conduction system of the developing heart at 11-12 days of development (HH41 to HH42) (Thomas et al., 2001).

The cell adhesion molecules cadherins are important for tissue organization during embryonic development. N-cadherin is involved in cell assembly and rearrangement during embryogenesis and is crucial for cardiovascular development (Linask, 2003; Kostetskii et al., 2005). N-cadherin is expressed in mesodermal tissues that differentiate into heart and is localized within heart forming regions (Linask, 1992). Blocking of N-cadherin in chick embryo at early pre-somatic stages (HH5) resulted in interrupted fusion of bilateral epimyocardial layers, thus not forming a single cardiac tube (Nakagawa and Takeichi, 1997). At the cellular level, large gaps between cells were observed where antibody against N-cadherin was injected. However some beating in these disorganized myocardial layers was observed suggesting that differentiation to cardiomyocytes was not altered but their contraction was not coordinated (Nakagawa and Takeichi, 1997). Also studies with targeted silencing of GATA4 revealed that N-cadherin expression in the middle part of the cardiac crescent is crucial for subsequent fusion of pericardiac cells and formation of a single heart tube (Zhang et al., 2003). Studies *in vitro* demonstrate that Msx1 and Msx2 (Hox8) regulate N-cadherin mediated adhesion and Msx1 is involved in cell sorting and proliferation (Lincecum et al., 1998). Msx1 is a transcription factor involved in heart morphogenesis (Chan-Thomas et al., 1993; Chen et al., 2007); it has not been described as a heart specific gene for the avian embryo, but it is expressed ectopically in the heart forming regions of the VAD quail embryo (Chen et al., 1995). Moreover, mutant Msx1-/- mice do not have any cardiac defects (Satokata and Maas, 1994) but Msx1-/-; Msx2-/- double mutant mice display a broad range of outflow tract malformations (Chen et al., 2007).

Fibronectin is one of the ECM proteins which along with other ECM compounds assembles already at early morphogenesis (reviewed in Rozario and DeSimone, 2010). The loss-of-function phenotype of fibronectin has been studied in mouse (George et al., 1993), *Xenopus laevis* (Davidson et al., 2006), zebrafish (Trinh and Stainier, 2004) and chick embryos (Linask and Lash, 1988a,b); it leads to embryonic lethality and,along with other defects, results in cardia bifida, defects in

mesoderm specification, myocardial precursor migration and yolk sack vasculature. Similar effects are obtained from studies where embryos are treated with exogenous RA (Osmond et al., 1991).

Retinoic acid receptors and retinoid X receptors are the key molecules in RA signaling essential throughout the development and for the maintenance various body functions. RARs and RXRs each have three subtypes. Studies with the VAD quail model demonstrate the critical time window at 4-5 ss when RA presence is crucial (Kostetskii et al., 1998) and RARα2, RARγ and RXRα are essential for RA signal transduction (Cui et al., 2003). The absence of RA signaling at that time leads to death of the avian embryo at 3.5 days as a result of multiple gross abnormalities that include retarded growth, abnormalities in central nervous system and the skeletal structure, the lack of circulatory system, and an abnormal, non-compartmentalized and dilated heart that lacks the inflow tracts and is oriented randomly (Zile et al., 2000; Zile, 2004).

An important part of cardiac morphogenesis is the establishment of the correct asymmetry because the subsequent process of looping (when the heart tube begins to form an S-shaped structure) is altered. The asymmetry molecular pathways are set up early in development and are regulated by a balance between transient stimulatory and inhibitory signals involving many genes at specific stages. The chick homolog of the mouse gene Nodal is expressed asymmetrically along the left side of the axis during and after gastrulation of the chick embryo (Levin et al., 1995; Zile et al., 2000). The transcription factor Pitx2 is also expressed in the left lateral plate mesoderm during early heart development and it has been linked to the regulation of L/R asymmetry pathway for heart and viscera. Pitx2 appears to be regulated by Nodal (St. Amand et al., 1998). Among many asymmetry genes is also the fibroblast growth factor 8, FGF8, which has been suggested to be a determinant for right-side asymmetry; one of its functions is to repress Nodal and Pitx2 on the right side of the embryo (Schlueter and Brand, 2009).

The molecular mechanisms that regulate early cardiac morphogenesis, however, are still not completely elucidated. In contrast, the use of knock-out mice has allowed the identification of proteins involved in events after the primary heart tube has developed, i.e. heart looping, epithelial-mesenchymal transition, heart segmentation into chambers, chamber maturation as well as the subsequent events leading to the development of a mature cardiovascular system (Nemer, 2008). The genes discussed in this section are only a small part of the molecular regulators of early heart development. Various interactions among transcription factors, signaling pathways, morphogens and other molecules are involved in the complex development of the cardiovascular system.

1.3.4. Early development of the vascular system

The principal components of the cardiovascular system include the blood, blood vessels, heart and the lymphatic system. Early heart morphogenesis has been described in the previous chapter. It does not take place without development of vascular system which is responsible for the distribution of primary blood cells and later for the distribution of oxygen and nutrients as well as removal of carbon dioxide and other metabolic waste products (LaRue et al., 2003; Eichmann et al., 2005). Development of a rich vascular network also ensures that every cell in the growing organism will receive adequate levels of oxygen and metabolites to meet its energy and structural requirements. Morphologically the structure of blood vessels is relatively simple. The largest surface of the vascular system is capillary bed (*tunica intima*), composed only of endothelial cells which are first to form during development. These capillary tubes are surrounded by a basement membrane; the larger vessels have additional layers that form the vessel wall that consists of a muscular layer (*tunica media*) and outer connective tissues (*tunica adventitia*) containing a network of small blood vessels (*vasa vasorum*) and nerves (Griendling et al., 2000).

Given the complex nature of the vascular system and variety of biological processes that are required for its formation and maintenance, it is no surprise that a large number of signaling pathways are involve in this process. Mutations in these pathways often manifest in phenotypes that result in embryonic lethality at mid gestation. Some of those mutation phenotypes have been described in mouse model systems. Notch, TGFβ, Sonic Hedgehog (SHH), VEGF and angiopoietin pathways are just some that if affected can lead to abnormal vascular development between E8.0 and E10.0 in mice, and ultimate embryonic lethality (Walls et al., 2008). The vascular activities of these pathways are not restricted and limited to this developmental window, they extend to organogenesis, maintenance of vascular homeostasis in adulthood and states of pathological angiogenesis. Some of the genes, e.g. Notch, TGFβ and SHH, are involved also in other developmental processes and are not restricted to vascular development.

The vascular system in any organ and tissue must be established early during development, since the diffusion distance of molecules is limited (for O_2 it is 100-200μm). The early development of the vascular system consists of vasculogenesis and angiogenesis. Angiogenesis is the formation of blood vessels from endothelial cells of pre-existing vessels, while vasculogenesis is the *de novo* formation of blood vessels from mesoderm (Weinstein, 1999; LaRue et al., 2003; Eichmann et al., 2005). Vasculogenesis is tightly correlated with heart development and it occurs simultaneously with formation of primary and secondary heart fields at the time when somites begin to form (Weinstein, 1999; Patan, 2000). Endothelial cell differentiation starts during gastrulation when cells

move through the primitive streak to form the mesoderm. Newly formed mesodermal cells organize into axial mesoderm (will give rise to the notochord) (Fig.1.15), paraxial mesoderm (somites) and intermediate mesoderm (kidney and gonads). The lateral plate mesoderm lies on both sides of the intermediate mesoderm.

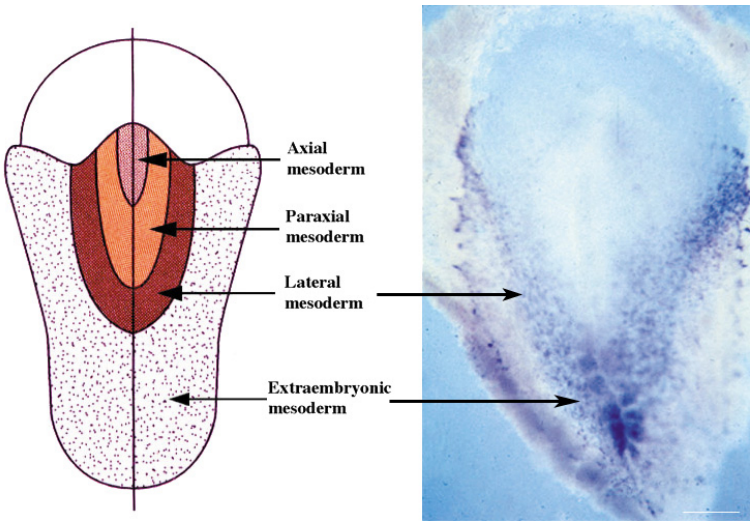

Figure 1.15. Origin of endothelial cells and distribution of mesodermal tissues in the chick embryo (gastrulation at stage HH4). The presumptive extra-embryonic and lateral mesoderm expresses VEGF receptor 2 (VEGFR2). Slightly modified from Eichmann et al., 2005.

The dorsal part of lateral mesoderm will develop contact with ectoderm and will form the body wall and limbs while the ventral part in contact with ectoderm will form visceral organs. The posterior part of mesoderm occupies half of the embryo during gastrulation and will give rise to the extra-embryonic mesoderm (Eichmann et al., 2005).

The peripheral cells in these mesodermal layers acquire the morphology of endothelial cells. The clumps of primitive mesodermal cells remaining adherent to the newly formed vascular endothelium, called blood islands, are the site of origin of extra-embryonic hematopoiesis. An ancestral progenitor common for both endothelial and hematopoietic cell lineages was postulated as angioblast and later renamed hemangioblast (see Tavian and Peault, 2005), suggesting it develops in yolk sac. The angioblasts contributing to the dorsal aorta arise by an EMT of cells originating from splanchnic mesoderm later in development. The endocardium arises from angioblasts that migrate some distance before assembly (vasculogenesis); the intersomitic arteries are the first vessels that form by angiogenesis and sprout off of the patent dorsal aorta (Poole et al., 2001). The vascular endothelial growth factor (VEGF), a heparin-binding, endothelial cell specific mitogen, is involved in vascular patterning and appears to be critical for migration, growth and morphogenesis of angioblasts into initial vascular patterns (Poole et al., 2001).

Hemangioblasts differentiate within the mesoderm partially in response to the signaling of fibroblast growth factor (FGF) family, but the mechanisms still are not clear, since they are involved in many other cell fate decisions. It has been reported that vascular endothelial growth factor receptor type 2, VEGFR2 (Flk1), plays a crucial role in differentiation of mesoderm exclusively to endothelial and hematopoietic lineages (Shalaby et al., 1997). VEGFR2 deficient cells are unable to contribute to the vascular network when placed in wild-type hosts, and accumulate in ectopic locations; this suggests that VEGFR2 is required for reception of signals that direct hemangioblasts towards their proper positions within embryo (Shalaby et al.,1997). The VEGF receptor 1, VEGFR1 (Flt-1) is also expressed exclusively in endothelial cells, but its disruption results in an increased number of mesodermal cells that become differentiated angioblasts; this increased density prevents cells from forming normal vascular channels (Fong et al., 1999; Patan, 2000). The functional differences between these receptors may reflect activation by different ligands within the VEGF family, i.e. VEGF-A and VEGF-B bind to VEGFR1, while VEGF-C and VEGF-D bind to VEGFR2 (Roman and Weinstein, 2000). In mice, an inactivation of a single VEGF allele causes embryonic death between days 11 and 12 due to malformation of the heart, undeveloped dorsal aorta and reduced number of primitive blood cells in the yolk sac, demonstrating that VEGF is required to maintain angioblast differentiation (Patan, 2000). TGFβ as well as FGF, VE-cadherin (vascular-endothelial cadherin) and N-cadherin have been linked to yolk sac hematopoiesis and/or vasculogenesis or angiogenesis, as is the case with cadherin molecules (Pepper, 1999; Bohnsack and Hirschi, 2004; Montero-Balaguer et al., 2009).

Hemangioblasts give rise to the blood islands which form the primary vascular plexus when fused (Fig.1.16). Blood islands consist of an internal core (hematopoietic precursors) and an external ring (endothelial cells).

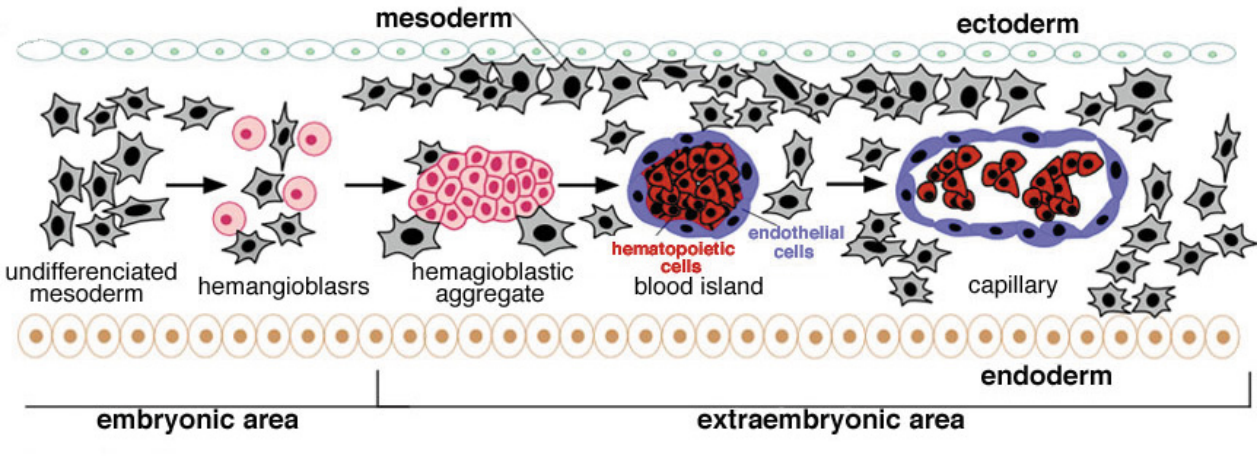

Figure 1.16. Schematic representation of differentiation of yolk sac blood islands (adapted from Eichmann et al., 2005).

Yolk sac hematopoiesis disappears after the 60th day of human development. Blood cells produced in the yolk sac are predominantly nucleated erythrocytes, which synthesize embryonic hemoglobin. This first pool of erythrocytes produced is known as primitive erythrocytes, and the blood cells produced later during development in liver are called definitive erythrocytes. Primitive erythrocytes have also been detected in the cardiac area as early as 3 ss (21 days of human development) indicating that vascular connections between the yolk sac and embryo are initiated at this early stage (Tavian and Peault, 2005). Inside the embryo, cells of the lateral mesoderm assemble symmetrically at the lateral sides of the embryo forming the pre-endocardial tubes, and then later fuse at the anterior intestinal portal. The fused region forms the endocardium of the heart. The formation of two ventral and two dorsal aortas is closely related to heart morphogenesis. The two dorsal aortas fuse later in development giving rise to one single vessel. The distal part of dorsal aortas gives rise to the *vitelline* or omphalomesentric arteries which fuse with the yolk sac vessels, similar to the two *vitelline* veins that form by splitting of the *sinus venosus* area (also called inflow tract) of the developing heart. Mesodermal cells of the extra-embryonic area give rise to the umbilical vessels (Patan, 2000).

Vasculogenesis results in the formation of major embryonic blood vessels and primary vascular plexus in the yolk sac. In the chick embryo, this takes place before 14 ss. The time when vascular plexus remodels into a system with arteries and veins is critical for embryo survival. Many mouse mutants for genes involved in vascular development die during this remodeling phase at the 14 ss (Roman and Weinstein, 2000).

Another event of vascular development is angiogenesis, the formation of blood vessels from pre-existing vessels. It consists of two processes: the sprouting of endothelial cells and the splitting of vessel lumens by intussusceptive microvascular growth (IMG). Organs that are derived from ectoderm-mesoderm, e.g. the brain and neuroectoderm, are vascularized by angiogenesis (Patan, 2000).

2. Materials and Methods

2.1. *in vivo* experiments

2.1.1. Quail embryo model

The generation of the vitamin A-deficient (VAD) quail embryo model and the criteria for normal and VAD quail embryonic development *in vivo* have been described (Dersch and Zile, 1993; Twal, et al., 1995). Briefly, normal embryos were obtained from eggs of quail (*Coturnix coturnix japonica*) raised on the Michigan State University Poultry Research and Teaching Farm and fed game bird chow (Purina Mills Inc., St. Louis, MO). VAD eggs were obtained from quail fed a semi-purified diet adequate in all nutrients except that vitamin A in the diet was replaced by all-*trans*-retinoic acid, which is not transferred to the egg (Dong and Zile, 1995). Eggs were collected daily and stored at 15°C until used. After incubation, the embryos were staged according to somite pair count and Hamburger & Hamilton (1951), using the standard avian HH stages to characterize the somite developmental stages (ss), as follows: pre-somite (HH4-6), 1 ss (HH7), 2-5 ss (HH8), 6-8 ss (HH9), 9-11 ss (HH10), 12-14 ss (HH11), and 15-17 ss (HH12, about 48 hr of incubation).

2.1.2.Treatment of embryos with all-*trans*-RA, or with antisense oligonucleotides to TGFβ2

Eggs were incubated horizontally for 26-34 h at 38.5°C and 60% humidity, windowed as described by Selleck (1996), and embryos at pre-somite (HH4) or 1-5 ss (HH7-8) were subjected to various treatments. VAD quail embryos were sub-blastodermally injected with 10-50 µl of Tyrode`s solution containing 10% VAD egg homogenate, 5% filter-sterilized Pelikan Fount India ink and a treatment substance, as described previously (Kostetskii, et al., 1998; 1999). In rescue experiments, 10 ng of RA were administered to the VAD embryos. This RA concentration had been demonstrated to be effective in the induction of normal cardiovascular development in the VAD embryo, without any teratogenic effects (Kostetskii, et al., 1998). Controls received the vehicle without RA.

For TGFβ2 gene blocking experiments, VAD embryos at 1-4ss were injected with 0.7 nmoles of TGFβ2 antisense oligonucleotides (AS). The chicken TGFβ2 AS sequence (GeneBank Accession no. X59080) is as follows: 5'-GCA CAG AAA TTG GCA TGA TAT CCT TTA GGT TC-3'. The oligonucleotides were synthesized with phosphorothioate modification at the Macromolecular Structure Facility at Michigan State University. Control embryos received either the vehicle alone or a non-specific oligonucleotide. After the various above treatments, the embryos (visible above the ink background) were staged, the eggs sealed and incubated for additional 6 to 40

h, the length of time dependent on the experimental design. Embryos were examined either for morphology, or analyzed for the expression of TGFβ2 and TGFβRII (TBRII) by *in situ* hybridization.

2.1.3. Whole-mount *in situ* hybridization

Normal and VAD embryos were dissected from extra-embryonal membranes, washed in cold PBS, and fixed in 4% paraformaldehyde (PFA) overnight at 4ºC. Embryos were dehydrated in a graded methanol series and stored at –20ºC until analysis. *In situ* hybridization was performed according to standard protocols using digoxigenin-tagged probes from available plasmids. Antisense riboprobes were prepared from chicken TGFβ2 (gift of the late Dr. Anita Roberts) and from chicken TBRII (gift of Dr. Joey Barnett). Synthesis of digoxigenin-tagged riboprobes was carried out with an *in vitro* transcription kit (Ambion) following the manufacturer`s protocol. The expression patterns of TGFβ2 and TBRII genes were examined at various early stages of development.

In situ hybridization was performed as described previously (Kostetskii et al., 1993). All embryos were re-hydrated before *in situ* hybridization. Hybridization was carried out at 60ºC overnight in standard hybridization solution containing 1 μg/ml dig-oxygenin-tagged cRNA probe. After NBT/BCIP reactions the stained embryos were examined either under microscope or embedded in paraffin and sectioned at 10 μm.

2.1.4. Immunolocalization of endothelial cells

Immunolocalization of endothelial cells was with the monoclonal antibody against QH-1 (Developmental Studies Hybridoma Bank, University of Iowa), a presumptive quail endothelial cell marker. Stage-dependent changes in endothelial cells surrounding the heart forming region were examined in normal and VAD quail embryos by peroxidase-biotin-streptavidin immuno-histochemistry. Embryos were fixed in 4% PFA, dehydrated, embedded in paraffin and sectioned at 10 μm. Deparaffinized and rehydrated sections were heated in citrate buffer (10 mM citric acid, pH 6.0) at 92–95ºC for 25 min for epitope retrieval. After cooling to RT, sections were treated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidase activity. Sections were next incubated in 1% bovine serum albumin (BSA)/10 mmol/l tris-(hydroxymethyl)-aminomethanebuffered saline (TBS), pH 7.4 blocking solution for one h to reduce non-specific binding of antibody, followed by incubation overnight at 4ºC with monoclonal antibody against QH-1, 1:100. Next, sections were incubated with horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG, 1:500 (Sigma) for 2

h at 4ºC. Antigen-linked peroxidase was detected with the chromagen 3-3'-diaminobenzidine (DAB) diluted in 10 m mol/l PBS, pH 7.2, containing 0.015% H_2O_2. Subsequently, the sections were lightly stained in Gill's hematoxylin, blued in 0.3% ammonium water, then dehydrated in ethanol-xylene and mounted with Permount. Antibody incubations were performed in Shandon racks; between steps the sections were rinsed with 10 mM TBS, pH 7.4.

2.1.5. Expression analysis by real-time PCR

To confirm the data obtained from whole-mount *in situ* hybridization, the expression of TGFβ2 was analyzed by quantitative (real-time) reverse transcription polymerase chain reaction (qRT- PCR) at the Center for Animal Functional Genomics at Michigaan State University. Primer sequences are shown in Appendix 1. The primers were designed using the Primer Express 2.0 program (Applied Biosystems). Total RNA was isolated from 4-7 ss normal and VAD quail embryos using TRIzol reagent according to manufacturer's protocol (Gibco) and subsequently cleaned using RNeasy kit (Qiagen). RNA was quantified using a UV spectrophotometer (A_{260}/A_{280}), and the quality of RNA assessed by agarose-formaldehyde gel electrophoresis. Total RNA (2.5µg) was reverse transcribed using oligo $(dT)_{12\text{-}18}$ primers and SuperScriptII-reverse transcriptase (Invitrogen), according to the manufacturer's protocol. Gene expression levels were quantified on an ABI Prism7000 (Perkin Elmer Corp., Applied Biosystems) using the Syber Green Universal Master Mix (Applied Biosystems). In brief, the reactions mixture (25 µl total volume) contained 20 ng single stranded cDNA, gene-specific forward and reverse primers (0.3 µM final concentration), 12.5 µl Syber Green RT-PCR Master Mix, and 10 µl ddH_2O, RNAse free. The real-time cycler conditions were as follows: PCR initial activation step at 95ºC for 15 min, 40 cycles each of melting at 95ºC for 15 sec and annealing/extension at 60ºC for one min. Assays were performed in duplicate using pooled RNA representative of 30 normal and 30 VAD embryos (30/each experiment). Relative fold-changes were quantified using the comparative CT ($\Delta\Delta C_T$) method (Winer et al., 1999). Data were normalized to the housekeeping gene β-actin and analyzed using the General Linear Model procedure in SAS statistical software (SAS Institute, Inc. Cary, NC; Version 7.0).

2.2. *in vitro* experiments

2.2.1. Cell cultures

Table 2.1. Overview on the cell cultures used in this work

Source	Description	Abbreviation	Reference
Chicken early stage embryos	heart forming region cell population	hfr	this work
Apical part of 42-day old chicken heart	heart mesenchymal cells	avian heart cells	this work
Human heart biopsy	heart mesenchymal stem cells	human heart MSC	kind gift of Dr.M. Hoogduijn Riekstina et al., 2009
Human umbilical cord	Human umbilical vein endothelial cells	HUVEC	Chemicon/Millipore
Human embryonic stem cell-derived cardiac progenitor cels	human cardiac progenitor cells	CPCs	kind gift of Dr.M.Puceat Blin et al., 2010
Mouse embryonic fibroblasts	Mouse embryonic fibroblasts	MEFs	kind gift of Dr.M.Puceat Blin et al., 2010

Table 2.2. Media used for the various cells

Name	Description	Cells cultured
DMEM	DMEM/10%FBS/1%p/s	avian hfr, heart cells, human MSC, MEFs
Endo	Endo-GRO LS (Chemicon/ Millipore)	avian hfr, HUVEC
α-MEM	α-MEM/10%FBS/1%p/s	human heart MSC
KO-DMEM	KO-DMEM/15%KO-SR	CPCs, MEFs

Avian heart forming region (primary) cell cultures

Freshly hatched normal chicken eggs were incubated for 36-42 h at 37°C. The embryos were dissected under microscope in cold phosphate buffered saline (PBS) and staged by their somite stage. Heart forming regions (hfr) from pre-somatic stage-2 ss, 3-5 ss and 7-14 ss were separated from the rest of embryonic tissue and kept in cold sterile DMEM with 1% penicillin/streptomycin and 2% fungizone (all from Invitrogen).

After dissection chicken hfr were washed with sterile PBS and treated with 0.25% trypsin-EDTA (Invitrogen) for 2-4 minutes. The primary cultures of 3-5 ss hfr cells were prepared from combined 10-15 explants treated with trypsin. One explant yielded ca. 50 000 cells. Trypsinized cells were counted and plated in 24-well plates, 20 000-30 000 cells/well and thereafter grown in

different media for three days at 37°C, 5% CO_2 in dark. Cardiac crest and underlying neural tube cells from the 3-5 ss embryo hfr explant produced a heterogenous cell population in culture. Explant cells developed adherent monolayer within three days in culture; the confluence of the samples cultivated in Endo medium was 30-40% , and in DMEM, 10-15%.

Standard DMEM base for hfr cell cultivation was supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (all from Invitrogen). Endothelial cell growth media base, Endo-GRO (Endo, Chemicon/Millipore), according to the manufacturer's protocol was supplemented with 2% FBS, 5 ng/ml Endothelial Growth Factor (EGF), 5 ng/ml basic Fibroblast Growth Factor (FGF), 15 ng/ml Insulin-like Growth Factor (IGF1), 50 μg/ml ascorbic acid, 1 μg/ml hydrocortisone hemisuccinate, 0.75 U/ml heparin sulfate, 10 mM L-glutamine (Chemicon/ MIllipore) and 1% penicillin/streptomycin (Invitrogen). Both media were supplemented with all-*trans*-RA (Sigma) to 10 nM (3 ng/ml) or100 nM (30 ng/ml) final concentration or with RA antagonist Ro 41 5253 (a kind gift from Hoffmann/LaRoche Co) to 0.5 μM, 1.5 μM or 3 μM final concentration. Control cells were grown without RA or Ro. All manipulations with RA were made in dark. Experiments were replicated five times with each concentration of RA and media.

Avian heart cells

Avian heart cells were obtained from apical part of 42-day old chicken heart. In brief, the apical part of the heart was minced and digested with 0.5 mg/ml sterile filtered collagenase type IV (Sigma). After two washes in RPMI media, dissociated tissue and cells were cultured in α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin (all from Invitrogen). Medium was supplemented with all-*trans*-RA (Sigma) to 66 nM (20 ng/ml) or 133 nM (40 ng/ml) final concentration and incubated at 37°C, 5% CO_2 for three, five and seven days. Control cells were grown without additional RA.

Human cells

α-MEM base for human heart mesenchymal stem cell (kind gift of Dr. Martin Hoogduijn) cultivation was supplemented with 10% FBS and 1% penicillin/streptomycin (all from Invitrogen). Human umbilical vein endothelial cells were grown in endothelial cell growth media base, Endo-GRO (Endo, Chemicon/Millipore), according to the manufacturer's protocol and was supplemented with 2% FBS, 5 ng/ml EGF, 5 ng/ml basic FGF, 15 ng/ml IGF1, 50 μg/ml ascorbic acid, 1 μg/ml hydrocortisone hemisuccinate, 0.75 U/ml heparin sulfate, 10 mM L-glutamine (Chemicon/ Millipore) and 1% penicillin/streptomycin (Invitrogen). Both media were supplemented with all-*trans*-RA (Sigma) to 66 nM (20 ng/ml) or 133 nM (40 ng/ml) final concentration and incubated at 37°C, 5% CO_2 for three, five and seven days. Control cells were grown without additional RA.

Human embryonic stem cell-derived early cardiovascular progenitor cells (CPCP) line has been recently characterized (Blin et al., 2010) and was used in these studies. In brief, human ESC were cultured in the presence of BMP2, then sorted after the SSEA-1 (CD15) and grown in defined media with certain supplements that result in the generation of endothelial cells, cardiomyocytes and smooth muscle cells. Human CD15+ cells and CD15- cells (kind gift of Dr. Michele Puceat) generated from HUESC-24 were cultured in 15% KO-SR in KO-DMEM (all from Invitrogen) cultured up to 7 days. The concentrations of all-*trans*-RA (Sigma) were as previously described for the avian cell cultures, respectively, 3 ng/ml (10 nM) and 30 ng/ml (100 nM). Control cells were grown without RA. All manipulations with RA were made in dark. Experiments were replicated five times with each concentration of RA and media.

2.2.2. Analysis of secreted TGFβ2 by ELISA

The amount of total secreted TGFβ2 protein in medium was quantified by TGFβ2 DuoSet ELISA (R&D Systems, Cat. #DY302; the antibodies in this kit react with avian proteins). In brief, medium was collected from incubated cells, and immediately used for ELISA. Media samples were incubated with 1 N HCl at RT for 10 min to activate TGFβ2 and neutralized with 1.2 N NaOH in 0.5 M HEPES. Assays were repeated five times with triplicate samples. The changes in secreted TGFβ2 in RA supplemented media were expressed as percent of secreted TGFβ2 from internal control (cells cultured in media without RA supplement). The mean values, standard deviation and statistical significance P values were calculated using GraphPadPrism v 5.0 software.

2.2.3. Expression analysis by PCR and real-time PCR

RNA from chicken hfr and heart cell cultures as well as from human cell lines was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. First strand cDNA synthesis was performed on 0.5-1 μg total RNA using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas). The quality of cDNA was tested in PCR reaction (25 cycles) with chicken housekeeping gene β-actin or human housekeeping genes β-actin, GAPDH, and PGK1. Chicken gene VEGF-A, GATA4 and Nkx2.5 mRNA expression was tested in obtained cDNA by standard PCR reaction (Veriti, Applied Biosystems): initial activation at 95°C 5 min, followed by 30 to 35 cycles of melting at 95°C for 15 sec, annealing at 58 to 60°C for 20 sec and elongation at 72°C for 20 sec. All primer sequences and reference numbers are shown in table 3. Gene expression levels were quantified by real-time PCR on an ABI Prism 7300 (Applied Biosystems) using Maxima SYBR Green/ROX qPCR Master mix (Fermentas). In brief, the reaction mixture in 20 μl

total volume contained 10 - 15 ng single stranded cDNA, gene specific forward and reverse primers (0.4 μM final concentration), 10 μl SYBR Green Master mix. PCR conditions: initial activation step at 95°C 4 min, 40 cycles each of melting at 95°C for 15 sec and annealing/extension at 60°C for 40 sec. Primer sequences are shown in Appendix 1. Assays were performed in duplicate. Relative fold-changes were quantified using the comparative CT ($\Delta\Delta C_T$) method (Winer et al., 1999). Data were normalized to the housekeeping gene β-actin or PGK1 or GAPDH. All products were resolved on 1.8% agarose gel with 100 bp DNA ladder (Fermentas).

2.2.4. HPLC analysis

RA samples for HPLC were prepared essentially as described (Miyagi et al., 2001). Briefly, ca. 700 000 cells from combined explant tissue in 500 μl of phosphate buffered saline pH 7.4 were vortexed for 60 seconds with 500 μl ethanol, 225 μl 2 M KOH and 600 μl *n*-hexane. After centrifugation at 15 000 rpm for 5 min, upper phase and lower phase were collected separately. Lower (water) phase was vortexed with 450 μl 2 M HCl, 600 μl of *n*-hexane, centrifuged as previously and upper phase was collected. The fractions were evaporated; each residue was dissolved in 200 μl of *n*-hexane and subjected to analysis on Agilent 1100 HPLC system (Agilent Technologies).

All-*trans*-RA solution in DMEM with 10% FBS was used for calibration. RA was analysed with UV detector at 351 nm in 100 μl samples using a reverse-phase Zorbax Eclipse XDB-C18 column 4.6x150 mm, 5 μm with MeOH-0.3 % acetic acid (80/20 v/v) mobile phase or at λ 350 nm using Zorbax SIL column 4.6x250 mm, 5 μm with *n*-hexane : 2-propanol : acetic acid (1000 / 43 / 0.675 v/v/v) mobile phase at flow rate 1.0 ml/min at 25°C. Results were evaluated by a ChemStation Plus.

Eukaryotic cells are cultured in different media, which contain defined composition of salts, amino acids, vitamins and sugars (media base), supplemented most frequently with FBS to 10% final concentration and antimicrobial substances. Since defined media base, DMEM from Invitrogen or Endo from Chemicon/Millipore, does not contain any retinoids, the only unmonitored source of RA in hfr cultivation experiments could be FBS or chick embryo cells themselves. We analyzed the presence of RA in FBS lots, which were obtained from Invitrogen and Chemicon/ Millipore as well as in the chick embryo explants cells.

HPLC analysis demonstrated that in 10% solution of the FBS purchased from Invitrogen preparation there was 0.99 ± 0.02 nM RA, while FBS purchased from Chemicon/Millipore contained 0.92 ± 0.02 nM RA (Table 2.3**).**

Table 2.3. The analysis of all-*trans*-RA in media

	all-*trans*-RA	
	reverse phase	direct phase
DMEM (10%FBS)	0.96 nM	0.95 nM
after extraction	n/a	0.70 nM
Endo (2%FBS)	-	-
10% FBS (Invitrogen)	0.99 nM	0.94 nM
after extraction	n/a	0.64 nM
10% FBS (ChemiconMillipore)	0.92 nM	0.90 nM
after extraction	n/a	0.77 nM

All values are compared using standards of 10 nM and 100 nM RA

Recalculation of the measured RA concentrations to 100% FBS, gives values close to 10 nM, which corresponds to the average RA concentration of human serum, i.e. 1.5–3.0 ng/ml or ca. 5-10 nM. No detectable RA was found in chick embryo explant tissues.

Control media used in all experiments without extra RA supplement, contained FBS at concentration of 10% (in DMEM) and 2% (in Endo) and therefore had base levels of 0.96 nM RA and 0.1 nM RA, respectively. These levels are below the normal RA concentration in human plasma (De Ruyter et al., 1979; Eckhoff and Nau, 1990), thus the addition of 10 nM RA would be closer to its physiological levels.

3. Results

3.1. TGFβ2 is negatively regulated by RA during early heart morphogenesis in VAD quail embryo

3.1.1. Abnormal heart inflow tract development in the vitamin A-deficient quail embryo

Several morphological abnormalities caused by deficiency of vitamin A have been described in the vitamin A-deficient (VAD) quail model. Embryos lacking any form of vitamin A die by the day 4 of embryogenesis (Dersch and Zile, 1993; Kostetskii et al., 1999; Cui et al., 2003). The earliest gross developmental changes are evident at the time of formation of the cardiovascular system. These changes manifest as an abnormal, un-looped, randomly oriented (left/right) but beating heart. Extra-embryonal blood vessels and the central nervous system (CNS) are abnormal. These studies have provided strong evidence that the presence of vitamin A is essential for posterior heart (primary heart field) to form its linkages between heart and the extra-embryonal blood supply, i.e. the inflow tracts, which are closed in VAD embryos. Dersch and Zile (1993) showed that administration of endogenous retinoic acid (the active form of vitamin A) at a specific time of embryonic development, the 4-5 somite stage of HH8, can rescue the development of the VAD avian embryo and normalizes the development of the cardiovascular system.

The progression of the gradual morphological changes in the cardiac region between the heart forming regions of normal and VAD embryos is shown in Figure 3.1. In order to determine if the closing of the inflow tracts (IFT) is due to decrease of endodermal cells, we evaluated the morphology and endothelial contribution to the posterior heart forming regions using QH1 immunostaining of transverse sections throughout the developmental stages 6-7 ss, 8-9 ss and 10-11 ss. First, the immunostaining between N and VAD samples in all stages is of similar intensity suggesting that proliferation and differentiation are not altered in the epimyocardial and endodermal layers at the posterior heart forming sites of the VAD embryos. Instead, the defects in morphogenesis at this site may involve disturbances in other cellular events, e.g. a lack of the certain components required for building the inflow tracts or cell migration. Second, transverse sectioning gave a better insight into the abnormalities of cell layer positions. At the 6-7 ss, the developing heart of the VAD embryo (Fig.3.1 B) is similar to that of the normal embryo (Fig.3.1 A). Any differences, if visible in the whole-mounts, are due to individual differences between embryos. Normal heart development at 8-9 ss is shown in Fig.3.1 C. The primitive heart tube is localized

slightly to the right, but the two parts of the primary heart field (primordia) are almost completely fused (Fig.3.1 c1), and will form a normal heart tube later in development. Inflow tracts are wide open but still pointed more posteriorly.

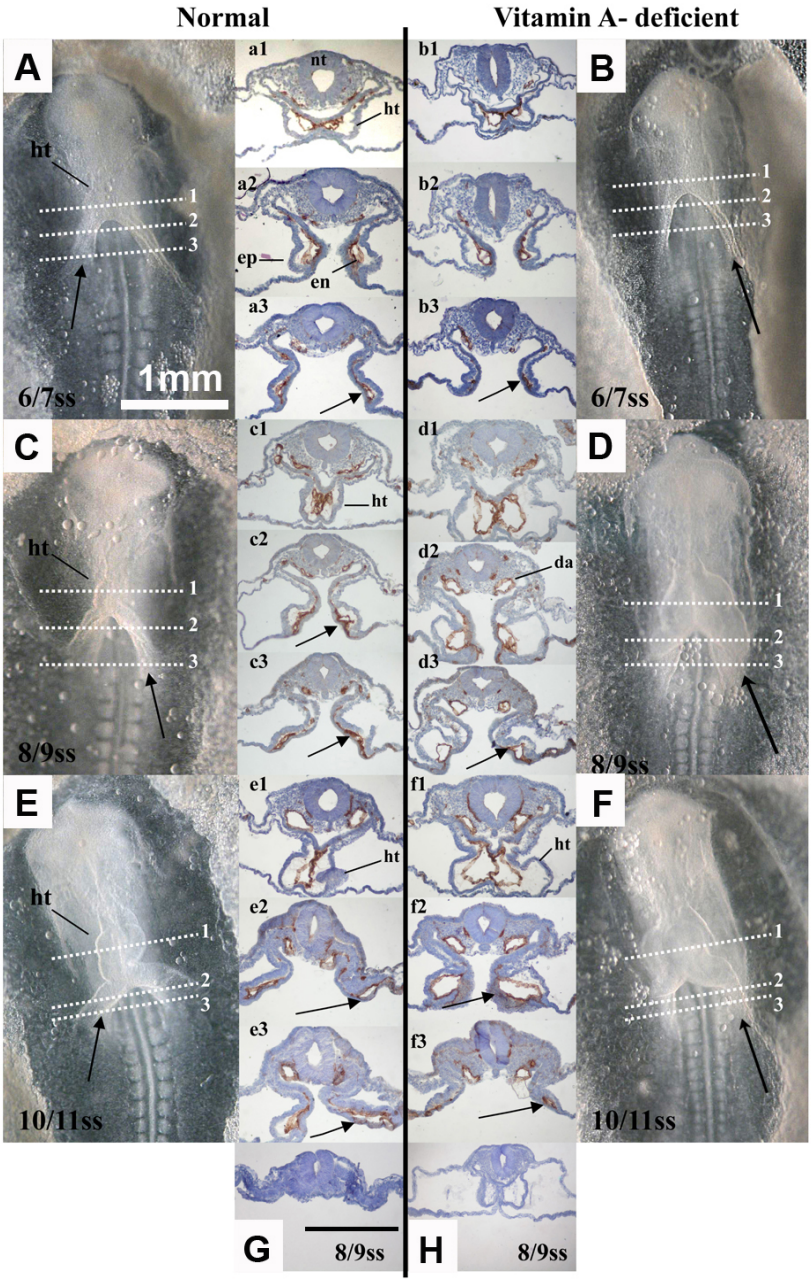

Figure 3.1. Morphology and QH-1 immunostaining of the posterior heart forming regions of normal and VAD quail embryos at 6/7 ss through 10/11 ss of development. Normal (A) and VAD (B) quail embryos at 6/7 ss are shown in whole-mounts and sequential transverse sections through the posterior heart forming region. QH-1 localization is associated with the endodermal cell layers. Normal development of 8/9 ss embryo is shown in (C); in the VAD embryo shown in (D) the inflow tracts (arrows) are abnormally expanded anteriorly but are beginning to narrow caudally (d3). At the 10/11ss, the normal embryo (E and e1) has a single heart tube (ht), positioned to the right, the inflow tracts (arrows) are open, linking the heart via the *vitelline* veins to the extra-embryonal circulation. The same stage VAD embryo is shown in panels F, f1, f2 and f3, revealing that the endodermal cell layers continue to be a part of the abnormally developing inflow tract structures in these embryos.
ss, somite stage; nt, neural tube; da,dorsal aorta; ht, heart; en, endocardium; ep, epimyocardium.
Arrows point to inflow tracts. Brown HRP staining localizes QH-1 in the cross sections. Scale bar in A applies also to B, C, D, E, and F; scale bar in G, 1mm, applies to all cross sections. Dotted lines in the whole-mounts indicate the sites of sectioning at approximately the same level in normal and VAD embryos. All views are ventral.

In the VAD embryo the inflow tracts are large and abnormal and beginning to narrow towards caudal ends at the 8-9 ss (Fig.3.1 D,d3). The heart is still in the middle, but primordia fusion is interrupted and epimyocardium is bulging ventrally (Fig.3.1 d1). At 10-11 ss the heart of a normal embryo is a single tube positioned to the right with a slight looping, the inflow tracts are wide open, positioned more distal from the midline and linked to the extra-embryonal circulation via *vitelline* (omphalomesentheric) veins (not visualized/marked in Fig.17). The heart of the VAD embryo shows visible morphological abnormalities. In addition to the previously shown morphological disturbances, i.e. the heart in the middle and bulging ventrally, we now clearly see that even at this stage the two endocardia are still not fused (Fig.3.1 f1). This is because the fusion of the heart tubes in the VAD embryos takes place later and eventually results in the formation of a single, ballooned, chamber-less heart (Dersch and Zile, 1993; Kostetskii et al., 1998; Zile, 2004). The IFTs in the VAD embryo are oriented caudally and more parallel to the midline. The endodermal cell layers continue to be a part of the abnormally developing IFT structures in the VAD embryo (Fig.3.1 f1,f2,f3). However, the endocardial and epimyocardial cell layers in these abnormally developing IFT structures of the VAD quail embryo are comparable to the normal. The structural deviation of the IFT from the normal, clearly manifested at the 10-11 ss, is likely due to differences in the architecture of the developing heart tube and foregut, affecting the morphogenesis of the IFTs, but not the differentiation of cardiomyocytes, since the heart is still capable of beating in VAD embryos, although weakly.

At this time it appears that the closing of the IFTs is due to the cellular layers from the opposite sides having moved together, resulting in the narrowing and subsequent total closure of the open spaces, thus cutting off the entry for the vascular links, i.e. the *vitelline* veins.

3.1.2. Over-expression of N-cadherin, TGFβ2 and its receptor TGFβRII in the absence of RA

N-cadherin is an important cell adhesion molecule that has been linked to heart morphogenesis (Kostetskii et al., 2005). It is expressed in the mesodermal tissue that differentiates into heart (Hatta et al., 1987) and is localized within heart forming regions (Linask, 1992).

Studies with 1-5 ss mice have provided descriptive analysis of TGFβ2 expression during early cardiac morphogenesis and show this gene to be expressed in the promyocardium of the *sinus venosus* of the inflow tract as well as in the foregut endoderm (Dickson et al., 1993). Earlier studies of Kostetskii and Zile (1993), suggested that if the early embryo lacks vitamin A, transforming

growth factor TGFβ expression is altered. TGFβ2 in the heart forming region (hfr) was the most altered isoform in VAD embryos. TGFβ2 in the avian embryo is expressed as early as pre-somite stages (HH4) and is localized in most tissues and in all cell layers (Jakowlew et al., 1994).

Here we examined the changes in N-cadherin and TGFβ2 gene expression using real-time PCR (Fig.3.2).

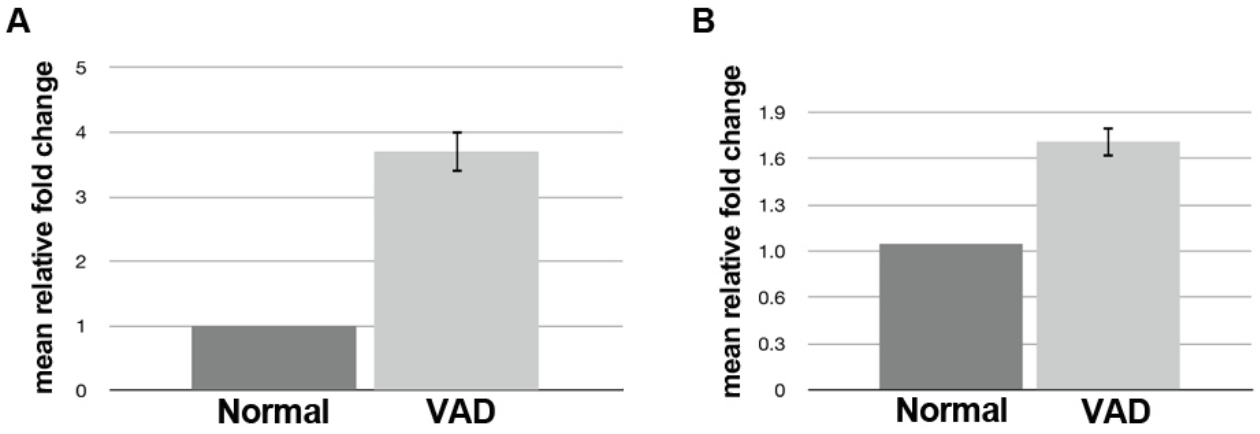

Figure 3.2. Quantification of differences in the expression levels of N-cadherin (A) and TGFβ2 (B) between normal and VAD embryos using real-time PCR, illustrating N-cadherin and TGFβ2 abundance in VAD embryos.

Quantification of the expression of both genes by real-time PCR indicates a significant ($p = 0.0002$) 3.7-fold change of N-cadherin and 1.7-fold change of TGFβ2 in 4-7 ss VAD embryos compared to the same stage normal embryos (Fig.3.2 A, B). A more detailed analysis of N-cadherin is described in Romeih et al. (2009).

The spatiotemporal expression of TGFβ2 in quail embryos was examined by *in situ* hybridization. At the 6-7 ss, there is an intense over-expression of TGFβ2 in the VAD embryos when compared to normal (Ghatpande et al., 2010). Cross-sectioning at the posterior level of the heart-forming region reveals that in both normal and VAD embryo TGFβ2 is expressed in all cell layers, but is over-expressed in the VAD embryo. At the 10-11 ss the intensity of the TGFβ2 in the VAD embryo is beginning to decrease, but still having abnormal morphology, suggesting that after 10 ss the presence of RA is not critical for TGFβ2 expression.

The expression of TGFβ2 and one of its receptor TBRII, a component of the TGFβ signaling complex (López-Casillas et al., 1991; Massagué et al., 1998), is shown in quail embryos at 7 ss in Figure 3.3. The expression of TBRII and TGFβ2 at various stages was examined and demonstrated that the expression of TBRII mimics the expression of TGFβ2, and that their expression intensity and localization are similar. At the 7 ss, there is an intense over-expression of TGFβ2 in the VAD embryo (Fig.3.3 B) in comparison to normal embryo (Fig.3.3 A). Similar over-expression is observed in the case of TBRII (Fig.3.3 D and E).

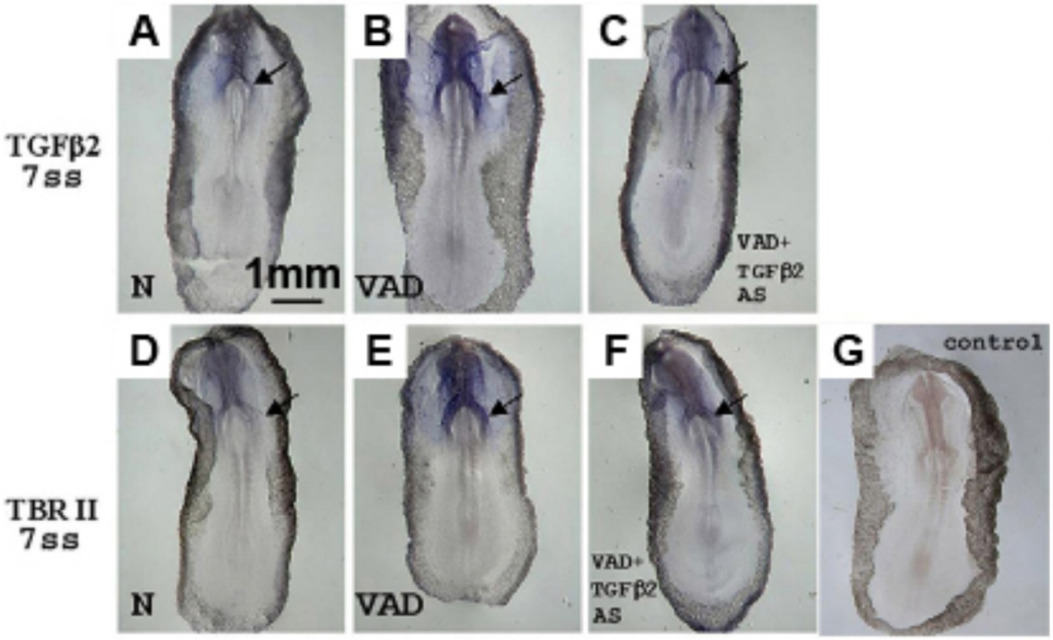

Figure 3.3. Expression of TGFβ2 and its receptor TBRII in whole-mounts of normal (N) and vitamin A-deficient (VAD) quail embryos at 7 ss (HH9) and blocking TGFβ2 and TBRII expression with anti-sense TGFβ2, assessed by in situ hybridization. The elevated levels of TGFβ2 between N and VAD embryos (panels A and B) and TBRII (panels D and E) are illustrated. Arrow points the posterior heart forming region. Treatment of VAD embryos at 1ss with TGFβ2 antisense oligonucleotide (0.7 nmol) normalizes both TGFβ2 (C) and TBRII (F) expression. G - negative control with non-specific oligonucleotides. All views are ventral.

This TGFβ2 over-expression in the VAD embryos continues at least until the 11 ss. This observation supports the involvement of the TGFβ2 signaling pathway in the RA-regulated heart morphogenesis. As shown by Ghatpande et al. (2010), cross-sectioning at the posterior level of the heart-forming region reveals that in both normal and VAD embryos TGFβRII (similarly to TGFβ2) is expressed in all cell layers; this is in general agreement with the data reported earlier (Jakowlew et al., 1994). This TGFβ2 over-expression in the VAD embryos continues at least until the 11 ss. This observation supports the involvement of the TGFβ2 signaling pathway in the RA-regulated heart morphogenesis. As shown by Ghatpande et al. (2010), cross-sectioning at the posterior level of the heart-forming region reveals that in both normal and VAD embryos TBRII (similarly to TGFβ2) is expressed in all cell layers; this is in general agreement with the data reported earlier (Jakowlew et al., 1994).

Additional support of TGFβ2 signaling pathway involvement in the RA-regulated heart morphogenesis comes from the finding that blocking the over-expression of the TGFβ2 in the VAD embryos normalizes not only the expression of TGFβ2 but also that of the TBRII. The administration of TGFβ2 antisense oligonucleotide to VAD embryos at 1 ss normalized the expression of both TGFβ2 (in 70% cases; n = 15) and TBRII (in 70% cases; n = 15) (Fig.3.3 C and F). The 7 ss was chosen for comparative evaluation of expression because at this developmental

time the differences in the expression in the TGFβ2 and TBRII genes between normal and VAD embryos are most pronounced, indicating peak transcriptional activity. Further results are described in greater detail in Ghatpande et al. (2010) (see attached copy of article) where it is shown that blocking excess TGFβ2 active protein rescues VAD cardiovascular phenotype whereas addition of active TGFβ2 protein induces the VAD phenotype in normal embryos.

3.2. Effect of RA on TGFβ2 and selected potential TGFβ2 target genes on chick heart forming region cells in culture

3.2.1. Development of cell culture model

My research and that of others in the lab of Zile (Ghatpande et al., 2010) suggested a role of RA as a negative *in vivo* regulator of TGFβ2 during early heart development of the avian embryo. To develop a model system for accessing the molecular mechanisms of regulation that are influenced by RA/TGFβ2 interplay during avian early embryogenesis, we established an *in vitro* system of chick heart forming region (hfr) cells.

To ascertain the functionality of the hfr ,we tested the viability of the explants from chick embryos in DMEM and Endo media. Chicken hfr explants were obtained after incubation of freshly hatched eggs. Eggs were opened and embryos dissected and staged according to somite stages (according to the somite count). The heart forming regions from 1-9 ss were obtained by dissection (Fig.3.4 A) and placed in separate wells on 24-well plate containing DMEM or Endo media and cultured for 24 h (Fig.3.4 B). Contractile cardiomyocytes were observed after a one day of cultivation in both DMEM and Endo medium.

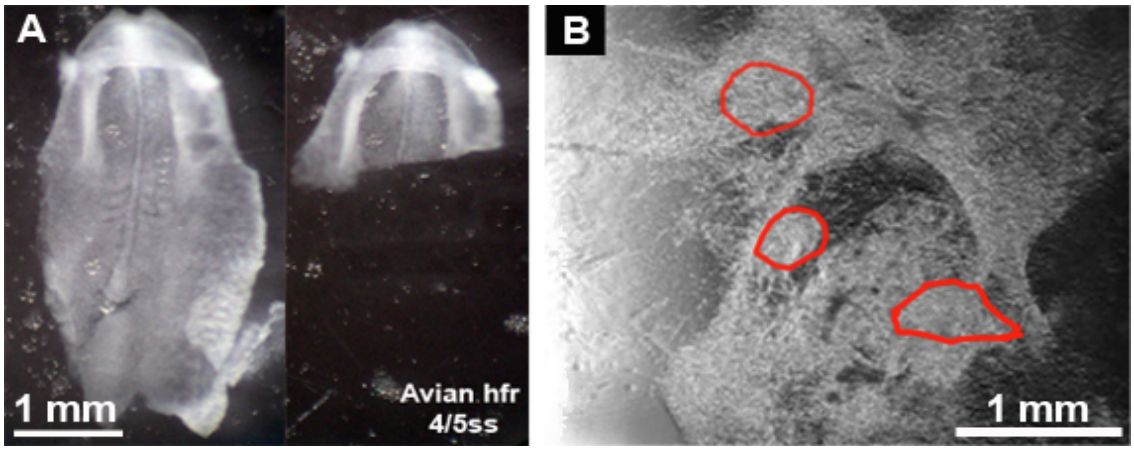

Figure 3.4. Chick embryo at 4 ss (A, left) and dissected heart forming region explant (A, right) after one day cultivation cultured in Endo medium (B). Red line marks the borders of contractile tissue developed within a day in culture.

Explants from all tested development stages developed contractile cardiomyocyte tissue aggregates when cultured in Endo media, however the yield of such tissue diminished at later stage explants, i.e. only one out of four at 7-10 ss. The development of contractile tissue in DMEM in comparison to Endo medium was poor at 1-2 and 3-5 ss; no contractile explants were obtained in DMEM at 7-10 ss (Table 3.1).

Table 3.1. Chick hfr explants from various embryonic development stages that were forming contractile tissue aggregates in cultivation media

	1/2ss	3/5ss	7/10ss
DMEM	1 (8)	1 (7)	0 (4)
Endo	3 (8)	7 (9)	1 (4)

Total number of explants placed in culture are shown in brackets.

All of the 7-10 ss explants cultured in Endo medium developed an adherent cell monolayer, while the same stage explants cultured in DMEM did not develop adherent cell monolayers. However, the media and culture conditions were not optimal for structural development.

VEFG-A, GATA4 and Nkx2.5 gene mRNA expression was determined to test the presence of cardiovascular cell population within hfr cell cultures.

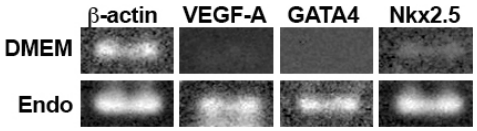

Figure 3.5. PCR analysis of chick hfr cell population cultured in two media. DMEM,DMEM, normal adherent cell growth media, Endo, Endo-Grow, endothelial cell media. β-actin was internal control.

Samples from Endo media gave stronger intensity bands for VEGF-A, GATA4 and Nkx2.5 gene mRNA transcripts in comparison to those from DMEM where VEGF-A and Nkx2.5 bands were very weak; no transcripts were observed for GATA4 **(**Fig.3.5).

Next, we tested the responsiveness of cultivated hfr cells to RA treatment. Homeobox gene HoxB1 is involved in early development of vertebrate embryos, and known to be regulated by RA both *in vitro* (Boncinelli et al., 1991) and *in vivo* (Wood et al., 1994; Maden et al., 1996). The accumulation of HoxB1 gene mRNA transcript amplification product from hfr cells grown in media with or without added RA at the end point of 40 cycle real time PCR is shown in Fig.3.6.

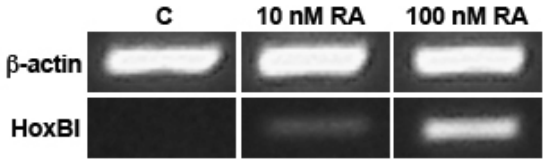

Figure 3.6. PCR analysis of the expression of HoxB1 gene mRNA in avian hfr cells grown in Endo media with RA supplement. β-actin was used as a reference in control (no RNA supplemented) and exogenous RA supplied cells.

The expression of HoxB1 was very weak in the control

samples of chick hfr cells that were grown in media without RA supplement. These results clearly demonstrate the stimulation of the HoxB1 gene mRNA synthesis by RA. Quantification of the differences of the concentration of the HoxB1 gene specific product revealed ca. 400-1000-fold increase of HoxB1 gene mRNA expression after RA stimulation.

3.2.2. Effect of exogenous RA on the secretion of TGFβ2 protein and the expression of TGFβ2 gene mRNA

Chick hfr cells were grown in DMEM and Endo media supplemented with exogenous RA at 10 nM concentration which corresponds to the average concentration of RA in human blood plasma, 5-10 nM (De Ruyter et al., 1979; Eckhoff and Nau, 1990) and at the elevated level of RA, 100 nM. Cell growth medium was collected after three day cultivation and used for TGFβ2 ELISA assay on the same day. Media without RA supplement was used as a control for each experiment.

Chick hfr primary cell cultivation experiments in RA-supplemented media were replicated five times.

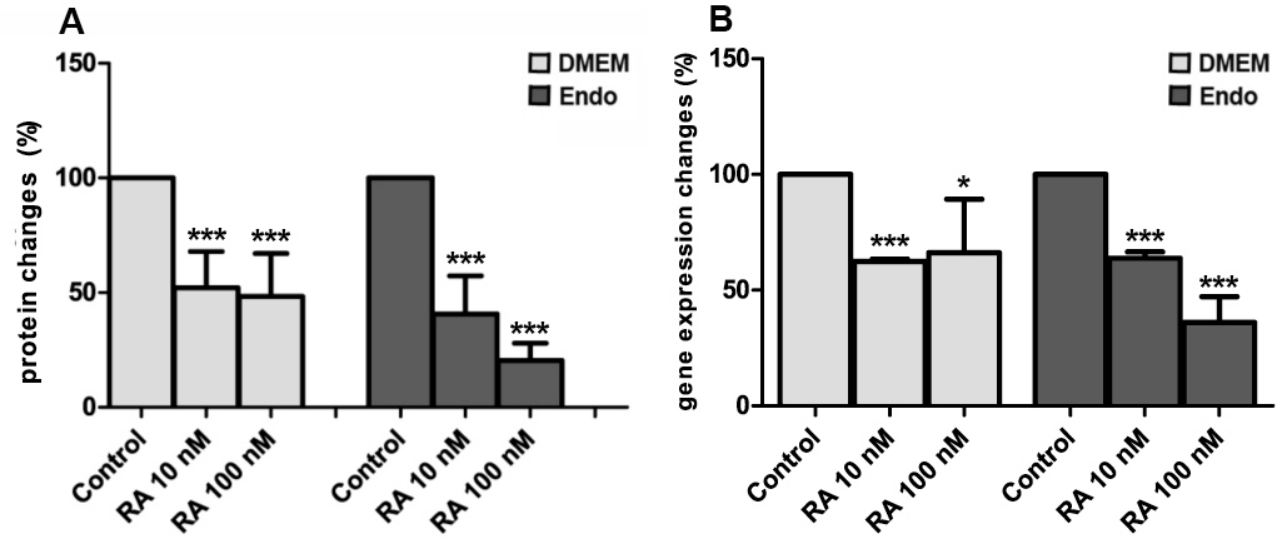

Figure 3.7. Influence of exogenous RA on TGFβ2 protein and mRNA synthesis in primary hfr cells grown in DMEM and Endo media for three days in culture. **(A)** Relative quantification of TGFβ2 total protein in the cultivation media (+/- per cent) in comparison to the control cells with no RA added (100%). **(B)** Relative quantification of TGFβ2 mRNA (+/- per cent) in comparison to the control cells with no RA added (100%). Standard deviation is indicated by error bars. The differences in comparison to the control at significance level $p<0.05$ are marked by (*), at $p<0.005$ by (**); at $p<0.0001$ by (***).

In comparison to the control without added RA, both added RA concentrations in a dose-dependent manner suppressed the accumulation of total TGFβ2 protein: protein concentration was decreased by ca. 50% in DMEM and by 60% and 80% in Endo medium (Fig.3.7 A).

TGFβ2 mRNA synthesis was also decreased in the presence of exogenous RA. The accumulation of TGFβ2 transcripts was reduced to ca. 65% in comparison to the control cells in

DMEM, and in dose-dependent manner to 60% and 40% level in comparison to the control cells in Endo medium (Fig.3.7 B).

3.2.3. Effect of exogenous RA on TGFβ/SMAD regulated gene and VEGF-A mRNA synthesis

To assess if the altered TGFβ2 mRNA synthesis and protein secretion in the presence of exogenous RA in hfr cells affects the expression of the mRNA of proteins that are regulated through the classical TGFβ/SMAD signaling pathway, we selected target genes according to previous studies in human fibroblast cultures (Verrecchia et al., 2001). The genes, which were identified in the above study, have been grouped in six clusters. We selected randomly one gene from each cluster (e.g. Fibornectin (FN1), c-myc, lamininB1, neogenin (NEO), CD44 and β-catenin). Since the decrease of the levels of TGFβ2 is observed using highest added RA concentration (100 nM), the genes were screened for their mRNA expression in chick hfr cell cultures grown in the presence of 100 nM RA (Fig.3.8).

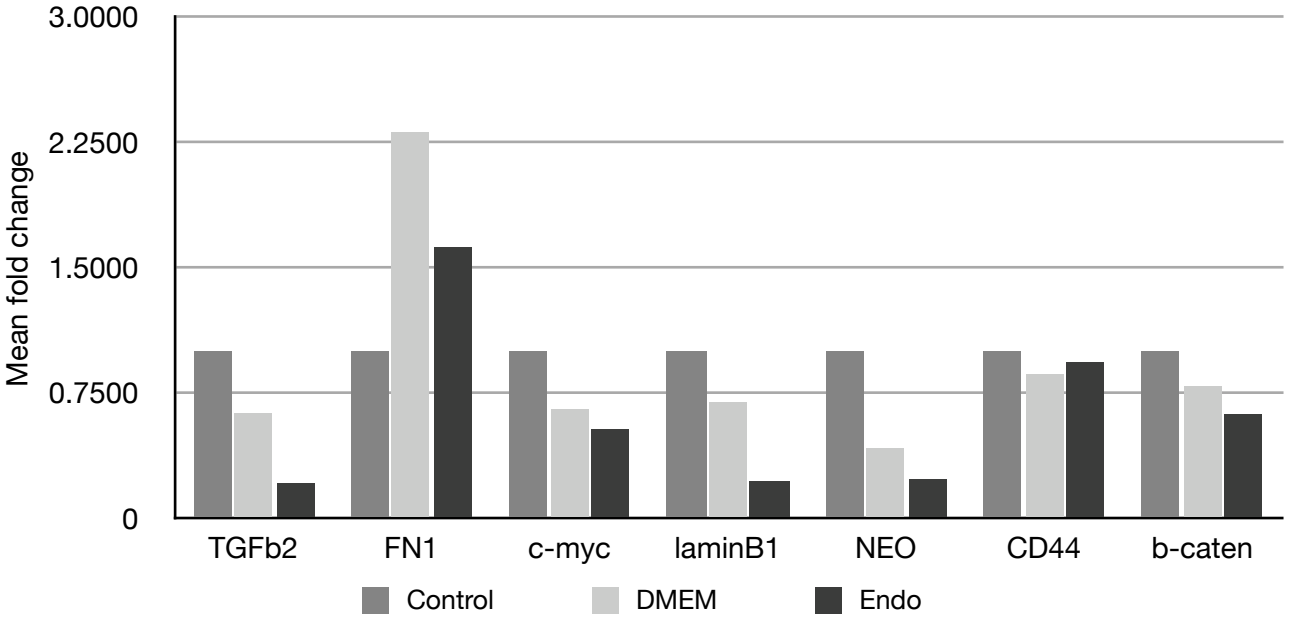

Figure 3.8. Relative quantification of TGFβ2 and TGFβ/SMAD target genes in DMEM and Endo media supplemented with 100 nM RA for three days.

TGFβ2 gene expression was greatly suppressed in the presence of 100nM RA; a significant decrease was also observed in the expression of laminin B1 (laminB1), neogenin (NEO), while decrease in c-myc and β-catenin (b-caten) expression was less pronounced and CD44 expression was not altered. In contrast, exogenous RA resulted in an greatly elevated expression of fibronectin (FN1) in the cultured primary hfr cells.

Next, it was of interest to examine the expression of FN1 and NEO in the primary hfr cell culture in response to different levels of exogenous RA. We choose FN1 because of its different from TGFβ2 response to RA, and NEO since its response to added RA was most pronounced.

FN1 encodes an extracellular matrix protein (Hay, 1981). In human cells it is significantly up-regulated by TGFβ2 through SMAD signaling pathway already at 30 min (Verrecchia et al., 2010). NEO gene encodes a multifunctional receptor that regulates many developmental processes, especially neural tube and mammary gland formation, myogenesis and angiogenesis (Cole et al., 2007). It is significantly up-regulated by TGFβ2 through SMAD signaling pathway in the human 2 h after TGFβ2 signal comes (Verrecchia et al., 2010).

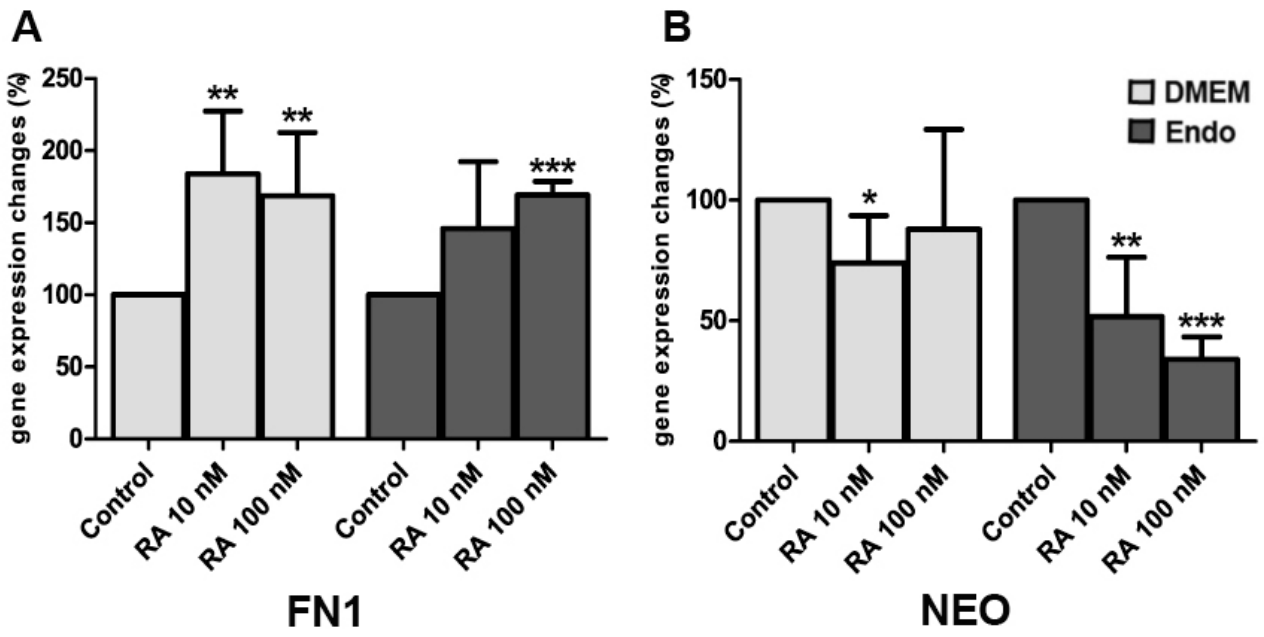

Figure 3.9. Influence of exogenous RA in primary hfr cells grown in DMEM and Endo media for three days in culture on the accumulation of mRNA of the genes that are regulated by TGFβ2 protein through SMAD signaling pathway. **(A)** Relative quantification of FN1 mRNA (+/- per cent) in comparison to control cells with no RA added (100%). **(B)** Relative quantification of NEO mRNA (+/- per cent) in comparison to control cells with no RA added (100%). Standard deviation is indicated by error bars. Differences in comparison to the control at significance level $p<0.05$ are marked by (*), at $p<0.005$ by (**); at $p<0.0001$ by (***).

FN1 gene transcript accumulation was stimulated by exogenous RA in both media. Cells from DMEM showed statistically significant, 84% and 68% RA dose independent increase of FN1 gene mRNA synthesis at both RA concentrations. Cells cultured in Endo medium showed statistically significant 63% increase of FN1 mRNA accumulation only at 100 nM RA, while the 46% increase at 10 nM RA in medium was not statistically significant (Fig.3.9 A).

Changes of NEO mRNA expression in the presence of exogenous RA paralleled those observed with TGFβ2 mRNA accumulation. mRNA expression levels of NEO decreased in the presence of exogenous RA. Changes of NEO gene expression in DMEM with supplemented RA

showed a tendency to decrease, which was impossible to confirm by statistical analysis due to high standard deviation of the data. More robust changes in the expression of NEO mRNA were observed in the hfr cells grown in Endo media, where expression was decreasing in RA dose-dependent manner to 51% and 34% level in comparison to the cells cultured without RA supplements (Fig.3.9 B).

Previous studies in the *in vivo* VAD quail embryo demonstrated impaired vasculogenesis along with other cardiovascular abnormalities in early development. VEGF plays a specific role in the maintenance and induction of growth of vascular endothelial cells (Ferrara et al., 1991) and also is a strong mitogen (Ferrara, 2000). Since the absence of RA results in a decrease of endothelial cell population and impaired vasculogenesis at 5-10 ss in the VAD quail embryo *in vivo* (LaRue et al., 2004), it was important to examine chicken Vascular Endothelial Growth Factor VEGF-A expression in the mixed hfr cell population *in vitro.*

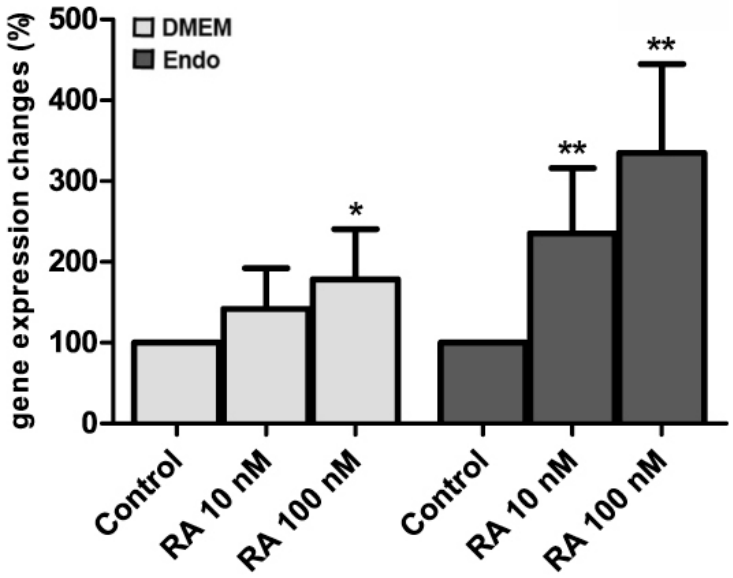

Figure 3.10. Influence of exogenous RA in primary hfr cells grown in DMEM and Endo media for three days in culture on the expression of the VEGF gene mRNA. Standard deviation is indicated by error bars. Differences in comparison to the control at significance level $p<0.05$ are marked by (*), at $p<0.005$ by (**); at $p<0.0001$ by (***).

Cells cultured in DMEM and Endo medium show an elevated, RA dose- dependent VEGF-A mRNA transcript accumulation (Fig.3.10). Hfr cells in DMEM with 10 nM RA supplement produced 42% and with 100 nM RA supplement,78% more VEGF-A mRNA expression in comparison to the control without added RA; the effect was even more pronounced in Endo medium, i.e. the increase was 135% at 10 nM RA and 235% at 100 nM RA.

3.3. Effect of RA deficiency on TGFβ2 and selected potential TGFβ2 target genes studied in chick heart forming region cells in culture

The RA antagonist Ro 41 5253 (Ro; Apfel et al., 1992) at 2-10 fold excess over physiological RA levels is known to block the binding of RA to RARα by inducing conformational change in the receptor (Keidel et al., 1994). At a 50-100 fold excess of Ro over RA also blocked is RARα mediated signaling. Ro has been used to mimic VAD status in studies of chick embryogenesis (López et al., 1995; Stuckmann et al., 2003).

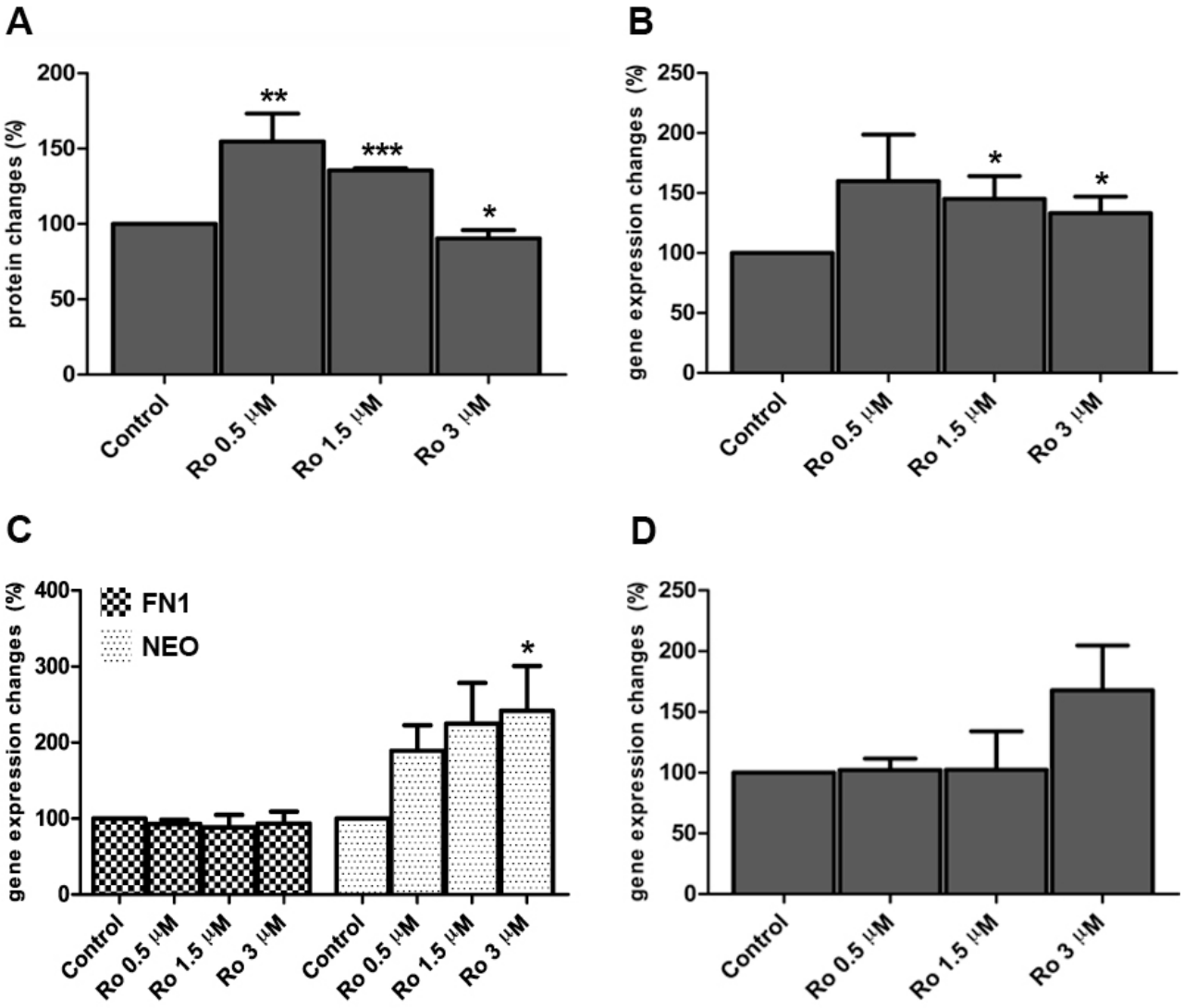

Figure 3.11. Influence of Ro on protein and mRNA synthesis in primary hfr cells grown three days in Endo media. **(A)** Relative quantification of TGFβ2 total protein in the cultivation media (+/- per cent) in comparison to the control cells with no Ro added (100%). **(B)** Relative quantification of TGFβ2 mRNA (+/- per cent) in comparison to the control cells with no Ro added (100%). **(C)** Relative quantification of FN1 (left) and NEO (right) mRNA (+/- per cent) in comparison to the control cells with no Ro added (100%). **(D)** Relative quantification of VEGF-A mRNA (+/- per cent) in comparison to the control cells with no Ro added (100%). Standard deviation is indicated by error bars. The differences in comparison to the control at significance level $p<0.05$ are marked by (*), at $p<0.005$ by (**); at $p<0.0001$ by (***).

FBS, which we used in our experiments, contained a typical concentration of plasma RA, which was correspondingly diluted by adding 10% or 2% of the serum to the cell growth medium. We tested the effectiveness of Ro in chick hfr cell primary culture to assess if the changes in protein expression will parallel those that are observed in VAD quail embryos *in vivo* (Ghatpande et al., 2010). Experiments were done in Endo medium only, since in the previous assays we found more clear-cut responses to RA in Endo medium as compared to DMEM.

All mRNA relative quantification experiments were repeated twice with 0.5 μM Ro concentration, three times, with 1.5 μM and 3.0 μM Ro concentrations. In the presence of RA antagonist Ro at 0.5 μM concentration the accumulation of TGFβ2 protein is increased by 54.9% in comparison to the control without antagonist. The increase tends to diminish when higher concentrations of antagonist are added: +35.7% at 1.5 μM Ro and 10% at 3.0 μM Ro (Fig.3.11 A).

TGFβ2 mRNA synthesis in response to the Ro treatment parallels the pattern of protein accumulation. At 0.5 μM Ro, TGFβ2 transcript accumulation is 60% higher, at 1.5 μM Ro, it is 45% higher and at 3.0 μM Ro it is 33% higher than in control cells without antagonist (Fig.3.11 B).

The antagonist does not have any influence on the expression of FN1 mRNA synthesis, while NEO transcript level is 80% elevated at 0.5 μM Ro concentration, 125% elevated at 1.5 μM Ro concentration and 140% elevated at 3.0 μM Ro, in comparison to control cells without antagonist (Fig.3.11 C). No changes in VEGF-A mRNA synthesis in response to RA antagonist were detected. At 3.0 μM Ro concentration the level of the VEGF-A expression is 69% higher than in the control cells, but the differences are not statistically significant (Fig.3.11 D).

3.4. Effect of excess RA on TGFβ2 and selected potential TGFβ/SMAD regulated genes in human cell cultures

3.4.1. Characterization of human heart mesenchymal stem cells

Recent data (Ghatpande et al., 2010; Li et al., 2010) and this work demonstrates that RA-regulated TGFβ2 expression and synthesis are elevated in absence of RA and decreased in presence of the exogenous RA in avian cells. The same interplay between RA and TGFβ isoforms have been shown previously in mouse embryogenesis (Mahmood et al., 1992; 1995). We next wanted to see whether RA elicits the same response on TGFβ2 signaling in human cells.

Adult stem cells are capable of self-renewal, and can give rise to different cell types. These cells are responsible for maintenance of homeostasis of our body tissue (Freitas and Dalmau, 2006).

Human heart mesenchymal stem cells were characterized by immunocytochemistry, flow cytometry and PCR methods using well described mesenchymal and embryonal stem cell markers (for details see attached copy of Riekstina et al., 2009) and the mesenchymal stem cells (MSC) from the heart were compared with other MSC derived from different tissues (Fig.3.12).

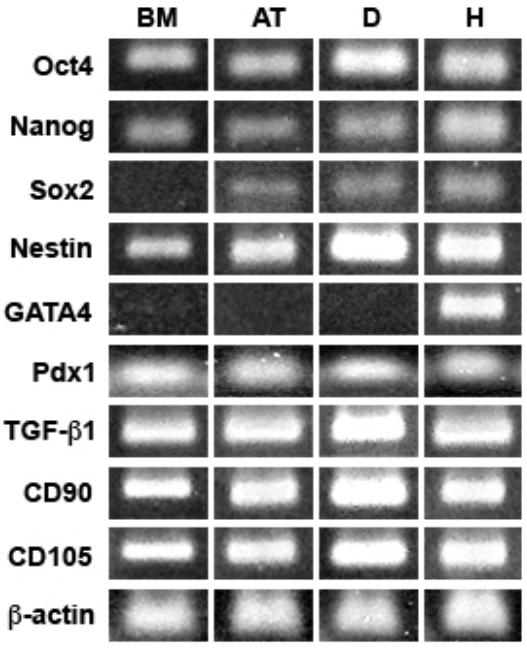

Figure 3.12. Semi-quantitative two-step PCR analysis of embryonic stem cell marker Oct4, Nanog, Sox2, and germ layer markers Nestin, GATA4, Pdx1 and TGFβ1, as well as mesenchymal stem cell marker Thy-1 (CD90) and Endoglin (CD105) expression in bone marrow (BM), adipose tissue (AT), dermal (D) and heart (H) mesenchymal stem cells. β-actin was used as internal control in all experiments.

Human heart cell samples express all the embryonic stem cell markers as well as germ layer markers that were chosen according to manufacturer's suggestions (RnD System's), where Nestin is used as ectodermal, GATA4 and Pdx1 as endodermal and TGFβ1 as mesodermal lineage markers. GATA4 is a cardiac specific marker expressed only in heart mesenchymal stem cells (Fig.3.12).

3.4.2. Effect of excess RA on the secretion and expression of TGFβ2 and its target genes in human heart mesenchymal stem cells and in avian heart cells

Cultured human heart MSC expresses all embryonic stem cell markers, and cardiac specific marker at the same time suggesting their "stemness". We next looked at the response of TGFβ2 to the presence of exogenous excess RA (66 nM and 133 nM) in cultured human heart mesenchymal stem cells (MSC) (Fig.3.13). The concentrations were chosen to be slightly above (40 ng/ml; 133 nM) and below (20 ng/ml; 66 nM) the concentration that is necessary for induction of atrial sub-lineage development (100 nM) in embryonic stem cells (Gassanov et al., 2009).

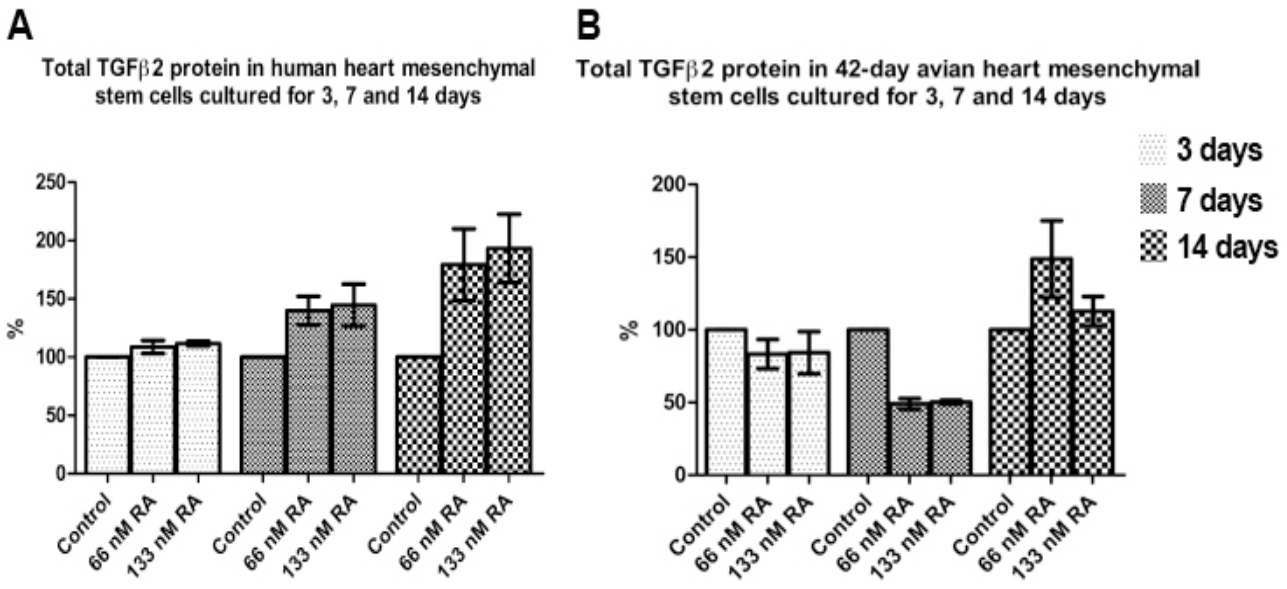

Figure 3.13. Total TGFβ2 protein in human heart MSC (A) and in 42-day avian heart cells cultured for 3, 7 and 14 days in media supplemented with 66 nM and 133 nM RA (X- axis). Total amount of TGFβ2 is compared to an internal control and given in percent. Bars indicate the standard deviation. Statistical significance, $p<0.05$.

Five human heart MSC lines were cultured in the presence of excess RA. In all of these cell lines there was a 39.8% and 44.5% increase of the total TGFβ2 in the 7-day cultures; this increase was highest when cells were cultured for 14 days, i.e. 79.2% and 93.3%, respectively (Fig.3.13 A).

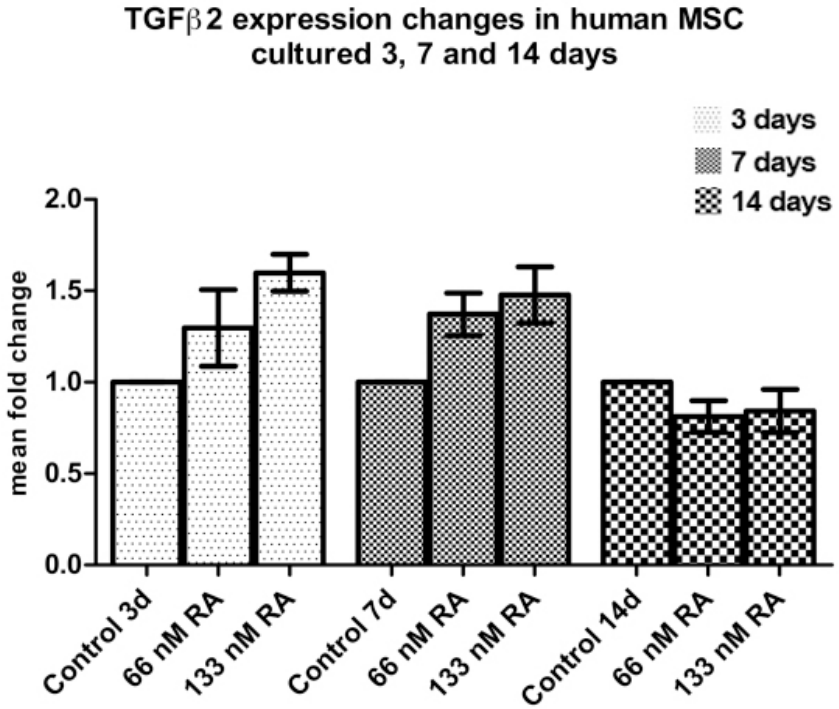

Figure 3.14. Relative quantification of expression levels of TGFβ2 in human heart MSC cultured for 3, 7 and 14 days in media supplemented with RA (66 nM and 133 nM ,on X- axis). Samples from RA–supplement-free media were used as controls (Control).

These results are not in line with the those obtained from *in vitro* experiments with chicken hfr cells cultured for 3 days, where in the presence of RA, the levels of total TGFβ2 are significantly decreased. To explore the observed differences, two adult chicken heart cell lines were tested for TGFβ2 response to the presence of exogenous RA (Fig.3.13 B). The results from 3-day and 7-day cultures demonstrate a 20% and 50% decrease of total TGFβ2 protein levels, respectively. In the 14-day cultures there were elevated levels of TGFβ2 protein, but these data are not statistically significant.

Data from real-time PCR (Fig.3.14) demonstrate 1.3-fold and 1.6-fold elevation in TGFβ2 expression levels in human heart MSC 3-day cultures, while the increases of 1.37-fold and 1.47-fold in 7-day cultures are not statistically significant.

In the previous chapter (3.2.4.) we described data from Verrecchia et al. (2001) of TGFβ/ SMAD regulated genes in human fibroblast cell cultures. The same genes from each cluster used for avian hfr cell screening were used for screening of five human MSC cell lines cultured in the presence of exogenous RA for 3 days (Fig.3.15 A), 7 days (Fig.3.15 B) and 14 days (Fig.3.15 C).

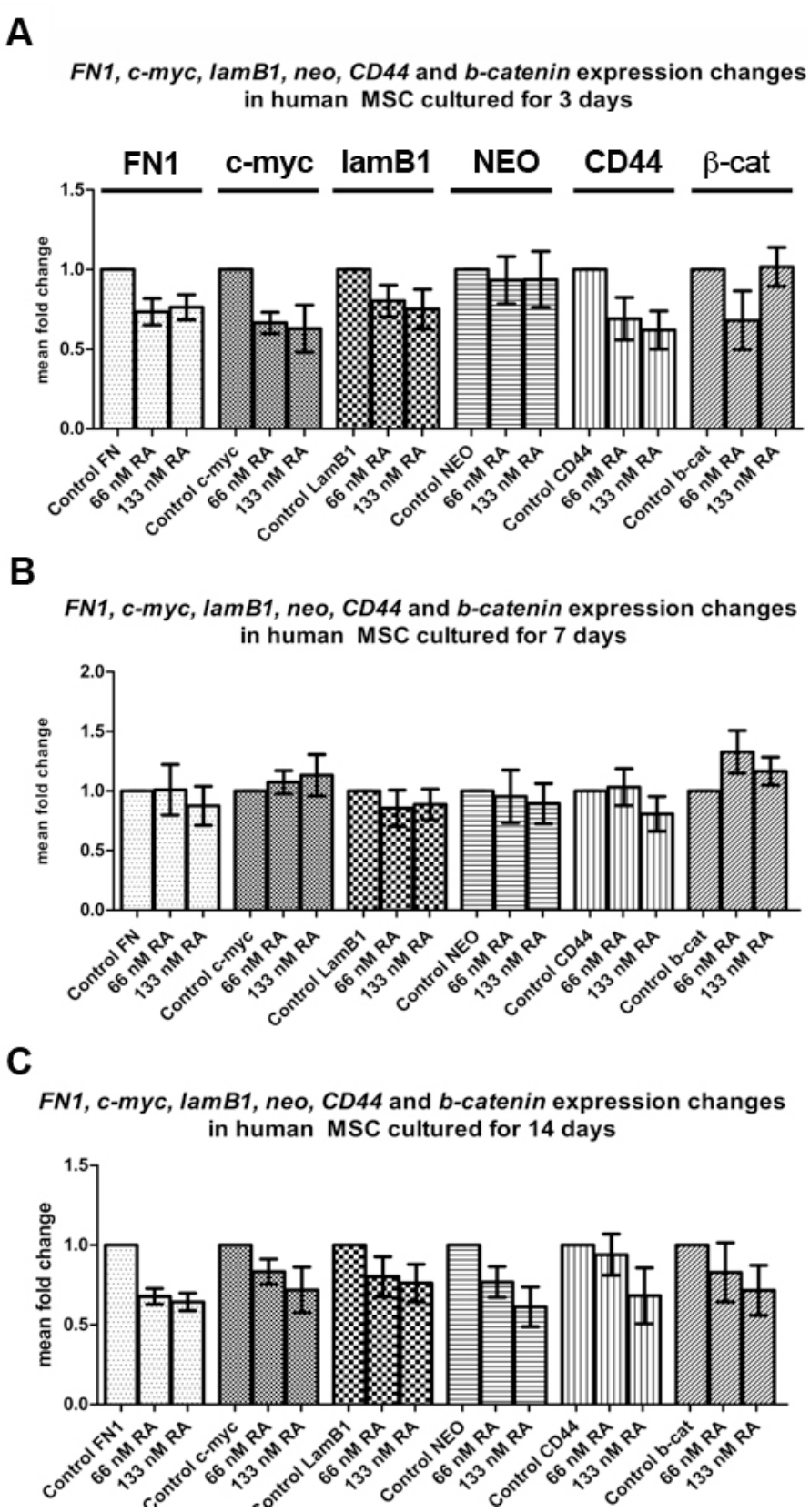

Figure 3.15. Mean fold changes in TGFβ/SMAD regulated gene expression in human MSC lines cultured for 3 days (A), 7 days (B), and 14 days (C) in the presence of excess RA. Bars indicate standard deviation.

In most of the results obtained from real-time PCR screening it was difficult to determine the effects of RA on gene expression due to the high standard deviations. Slight similarities between human

MSC and avian hfr cell culture results could be observed in the expression mean fold changes between TGFβ2 and FN1, the expression levels of which are decreased in the presence of RA. The expression of other genes is either down-regulated or not affected and mostly are statistically insignificant (Fig.3.15).

3.4.3. Effect of excess RA on the secretion of TGFβ2 protein in human umbilical vein endothelial cells

We next looked for vertebrate endothelial cells that would be closer in their developmental stages to those used from the avian embryos. To further examine the interaction between RA and TGFβ2, we obtained human umbilical vein endothelial cells (HUVEC) from Chemicon/Millipore and cultured these cells for up to 15 days in defined endothelial cell media (Endo), supplemented with 66 nM and 133 nM RA.

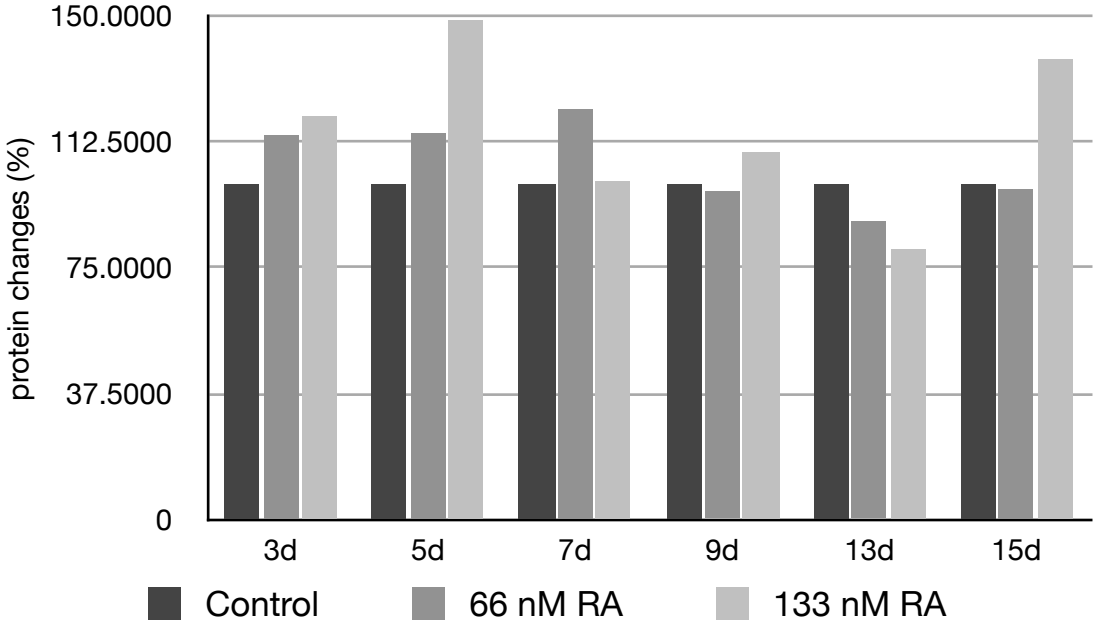

Figure 3.16. Total TGFβ2 protein in HUVEC cultured in Endo-Grow media supplemented with 66 nM and 133 nM RA and compared to control media without RA (100%). Cell media samples were taken every 2 to 3 days. Days of cells in culture are shown on X- axis.

The results show a slight increase in total TGFβ2 protein level at day 3 (Fig.3.16); the strongest response to the presence of excess RA is in the sample from day 5 in media with 133 nM RA (50% increase above control). By day 7 the levels of TGFβ2 protein are elevated in the presence of 66 nM RA but not in the presence of 133 nM RA, whereas at the day 15 there is approx. 35% elevation in total TGFβ2 protein.

3.4.4. Effect of exogenous RA on the secretion and expression of TGFβ2 in human cardiac progenitor cells derived from embryonic stem cells

In the studies described here we examined the role of RA in the regulation on TGFβ2 expression and protein level in various cells to determine if the responses to RA are similar between species (chicken and human) and cell types (heart MSC and endothelial cells). We have observed that RA can be either a positive or a negative regulator in the examined embryonic and adult cells. It was of interest next to examine human cells from early cardiovascular development, i.e. embryonic stem cells.

Fig.3.17 illustrates the results when CD15+ cells (CPCs), were tested for their response on TGFβ2 in the presence of added RA. While total TGFβ2 protein concentration was very low (299 pg/ml) in the cells grown in control media, total protein was elevated in cells grown in media supplemented with 100 nM RA (Fig.3.17).

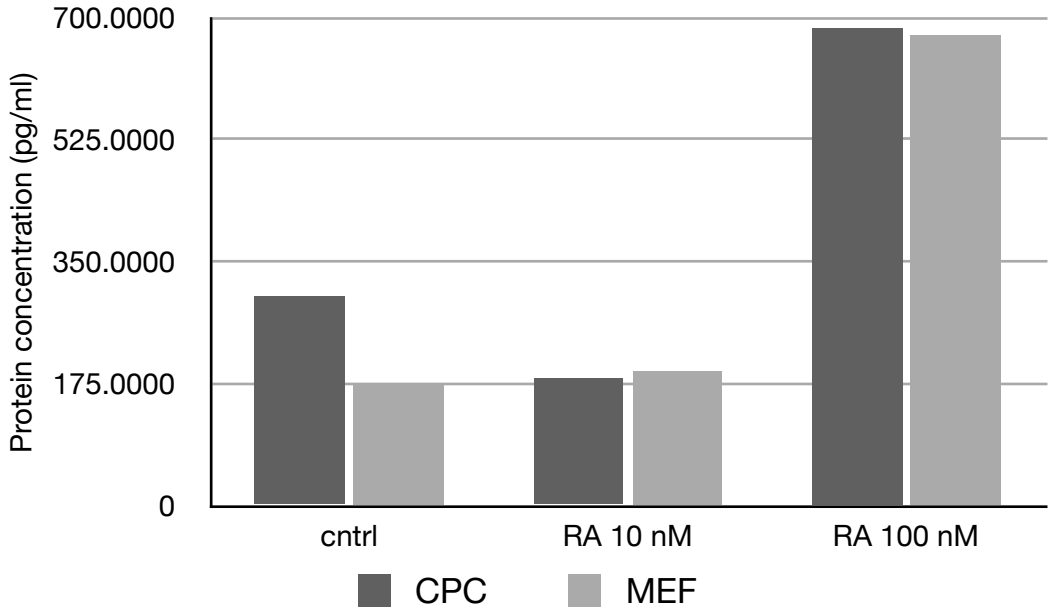

Figure 3.17. Total TGFβ2 protein concentration in CPCs (dark bars) and MEFs (light bars) cultured in media supplemented with RA (concentrations shown on X axis) for 6 days.

Next, we tested the mRNA expression levels of TGFβ2 and FN1 (Fig.3.18). RNA from MEF feeder layer grown in media without RA supplement was used as control for species-specific primers; no transcripts were detected in real-time PCR analysis; thus the data from this analysis show human CPC-specific response to the presence of excess RA. Expression analysis of TGFβ2 transcripts (Fig. 3.18) demonstrate a 0.23-fold decrease of the TGFβ2 transcription in presence of RA at

physiological concentrations (Roos et al., 1998) and only 0.6-fold decrease in the presence of excess RA.

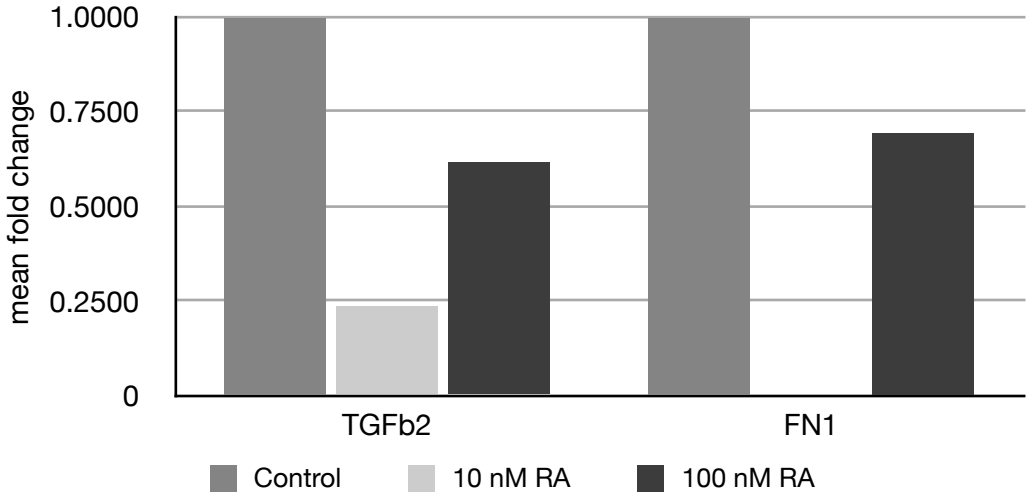

Figure 3.18. Relative quantification of TGFβ2 and FN1 expression in CPCs cultured in media supplemented with RA and cultured for 6 days.

The mean fold change data from samples of media with 10 nM RA show a 0.00072-fold decrease in FN1 expression, the presence of 100 nM RA results in a 0.7-fold decrease. The data presented here represent only one experiment; clearly, more CPC lines must be tested to obtain statistically significant data.

4. Discussion

4.1. Vitamin A deficiency leads to severe cardiovascular defects in VAD quail embryos

Retinoid deficiency during embryonic development causes abnormalities in organs of epithelial and mesodermal origin preventing the normal formation of cardiovascular system in the avian and mouse embryo (Heine et al., 1985; Dersch and Zile, 1993; 2004; 2010; Niederreither et al., 2001). It was discovered that in the avian embryo, at the 4-5 ss during neurulation, the presence of RA is essential for subsequent normal development (Kostetskii et al., 1998). If RA is administered to the VAD embryo during this time window, the embryo is rescued and develops normally. The cardiovascular system abnormalities caused by a complete lack of RA are characterized by an absence of vascular networks and by a ballooned, non-compartmentalized, randomly-positioned heart without an open inflow tract (IFT) at its posterior part (Heine et al.,

1985; Dersch and Zile, 1993; Zile, 2010). *In vivo* studies show that the basis for later cardiovascular defects is the failure of the primitive heart tube to stay open at its posterior end (inflow tract), thus preventing the formation of *vitelline* (omphalomesenteric) veins normally connecting the embryo with the extra-embryonal circulation (Dersch and Zile, 1993; Zile, 2004; see also Fig.3.1). The data from sequential steps in the formation of IFT (Fig.3.1) revealed that in the VAD embryo epimyocardial and endodermal cell layers are similar to those of normal embryos and that endothelial cells (marked with QH1 Fig.3.1) are present within their appropriate structures. There is study on the effects of vitamin A deficiency on numbers of endothelial cells and angiobalsts demonstrating the 40% decrease in numbers of endothelial cells within *sinus venosus* (IFT) area in VAD quail embryo at 10 ss (LaRue et al., 2004). Our data confirm the narrowing and closure of the IFT in VAD embryos by stage of 10 ss, which cut off the entry for vascular links.

Since the proliferation and differentiation in endodermal cell layers seems not to be altered, the defects in morphogenesis at these sites may be due to disturbances in other cellular events, e.g. a lack of certain molecules necessary for building the IFT. Our studies implicate an excessive N-cadherin expression (Fig.3.2.A) in the VAD quail embryos as a likely contributor to the abnormal IFT formation, since the over-expression of this molecule results in abnormal vascularization (Romeih et al., 2009). It is also possible that an excess of N-cadherin interferes with the organization of IFT. It appears, that only a strictly limited physiological amount of this molecule is required for normal development of IFT.

Various growth factors (TGFβ, VEGF, FGF) and and their signaling pathways are involved in early vasculogenesis and heart development; their interplay as well as RA regulation is essential for normal cardiovascular development (Poole et al., 2001; Wagner and Siddiqui, 2007a; Nemer, 2008).

4.2. Expression of TGFβ2 and TGFβRII is increased in the absence of RA

In mice, at days 6.5-9 post implantation (coincides with 1-5 ss in avian embryogenesis) the TGFβ2 isoform is mainly expressed in the promyocardium of the *sinus venosus* of the IFT and in the foregut endoderm (Dickson et al., 1993). Our work demonstrates that the expression patterns of TGFβ2 and its receptor TGFβRII in the normal and VAD quail embryos at the neurulation stage are similar; and transcripts are mainly restricted to the anterior part of the embryo, including the neural folds and the heart forming regions (Fig.3.3); expression is present in all embryonic cell layers.

These observations are consistent with the data of TGFβ2 mRNA and protein localization in chicken embryos (Jakowlew et al., 1994).

Our data demonstrate the over-expression of these genes due to absence of RA. The global expression of TGFβ2 gene is 1.7-fold increased in VAD embryos at 4-7 ss (Fig.3.2 B) suggesting that RA can regulate its expression. However, TGFβRII, an important component of the TGFβ signaling pathway (López-Casillas et al., 1991), is also over-expressed in absence of RA. This observation supports the hypothesis that TGFβ2 signaling is involved in RA-regulated cardiovascular development. The addition of antisense TGFβ2 to VAD embryo *in vivo* decreases and normalizes the levels of TGFβ2, and also normalizes the expression of TGFβRII (Fig.3.3). However, this finding does not prove that TGFβ2 and TGFβRII are an obligate receptor-ligand pair. Our data of TGFβ2 and its receptor response to the absence of RA differ from previously described data when the opposite process to deficiency is mimiced by addition of exogenous RA to cell cultures, where TGFβ2 expression is increased (Glick et al., 1989; Yoshizawa et al., 1998). There is also evidence of differential cell type-specific RA effect on TGFβ2 levels (Glick et al., 1991). These contradictory observations emphasize the complexity of the regulatory mechanisms at the pre-receptor level of TGFβ signaling, since activation and regulation of this signaling can occur at various levels. The localization of TGFβ2 and TGFβRII transcripts is also in the same cardiogenic cell layers as the localization of the transcripts of the RA receptors RARα2, RARγ and RXRα (Kostetskii et al., 1998; Cui et al., 2003), strengthening the hypothesis of an interaction between TGFβ2 and RA signaling during early cardiovascular development.

Our findings suggest that a negative regulation by RA of TGFb2 signaling protects the embryo from excessive TGFβ2 activity during critical developmental periods. While there is evidence that RA signaling is a negative regulator for certain malignant processes (Sun and Lotan, 2002), the role of RA as a negative regulator of TGFβ2 in the context of normal embryonic development was first demonstrated only recently (Ghatpande et al., 2010; this work).

In studies on potential downstream signaling pathways by TGFb2,we discovered that protein levels of pSMAD2 or pSMAD3 4 are not altered in VAD embryos, whereas Erk1/2 levels are increased (Ghatpande et al., 2010). Erk1/2 is a signal transduction molecule on the Ras/MAPK pathway. This observation suggests that TGFβ2 when elevated (as in the absence of RA), could transfer its signals through the MAPK pathway. In support of these observations are reports of TGFβ signal transduction through other than the canonical SMAD pathway, e.g. the MAPK pathway (Derynck and Zhang, 2003) or cross-talk between these two pathways (Lee et al., 2007;

Guo and Wang, 2009). However, the link between overactive TGFβ2 and overactive pErk2 during RA-regulated embryonic development remains to be unambiguously verified.

In addition, preliminary work from Zile lab (unpublished) identifies other genes that may be involved in heart formation and are differentially expressed in VAD embryos. Some of the genes have been suggested to be linked to TGFβ signaling, e.g. the EMT process-related extracellular matrix protein fibronectin (Mercado-Pimentel and Runyan, 2007), which is significantly up-regulated in VAD embryos during 6-10 ss, the time of IFT closure, suggesting a role for ECM in IFT formation. Another molecule, intersectin, is also elevated in VAD quail embryos in heart forming region (unpublished data from Zile lab). Intersectin is a multi domain adaptor protein and is involved in several signal transduction pathways, especially in the regulation of Ras induced activation of MAPK kinases (Adams et al., 2000).

In summary, in our *in vivo* studies we have made the following observations: 1) endogenous RA is a critical negative regulator of TGFβ2 *in vivo* during early avian embryogenesis; this regulation occurs at the transcriptional level; 2) the elevated levels of TGFβ2 due to an absence of RA result in closed heart inflow tracts thus making it impossible for extra-embryonal vasculature to link to the ~~the~~ heart tube; this demonstrates that strictly regulated TGFβ2 levels by physiological levels of RA are essential for normal IFT formation which is followed by the positioning of the heart tube to the right side and the subsequent looping of the heart.

4.3. Effect of RA on TGFβ2, VEGF and on TGFβ/SMAD target genes in avian heart forming region cells *in vitro*

Vitamin A deficiency and excess have profound effects on the development of the vertebrate embryo. These effects differ and are related to tissue-specific sensitivity to presence or absence of RA (Zile, 2004; Campo-Paysaa et al., 2008).

The abnormalities observed in studies with excess RA showed remarkable similarities to those generated by a lack of vitamin A, thus identifying common development pathways that are disrupted by an imbalance of vitamin A (Ross et al., 2000; Maden, 2001).

In our studies of effects of exogenous RA on chick heart forming region cells (primary cell culture) we use two concentrations: 10 nM RA that is close to physiological levels in plasma (De Ruyter et al., 1979; Eckhoff and Nau, 1990) and 100 nM that is slight excess and have been reported to cause some adverse effects on cardiac cell development in zebrafish (Waxman and Yelon, 2009). Furthermore, in our present studies other important experimental conditions must be

taken in account, i.e. the basic media with FBS always had a background level of RA that was near normal for embryonic cells (10% FBS had 0.96 nM RA and 2% FBS - 0.2 nM RA). We did not observe any morphological changes in cell cultures among different treatments. The experiments with RA antagonist were conducted to mimic complete RA deficiency in chick heart forming region primary cell cultures.

TGFβ2 is essential for the movements and formation of heart fields and cardiac crest *in vivo* during the very early embryogenesis (until 3 ss) and again later on, i.e. after 20 ss, during EMT and endocardial cushion formation (Table 4.1). The interplay between RA and TGFβ2 involves different cell types during avian embryonic heart morphogenesis. RA is required for many early developmental events, including vascular development regulating endothelial cells (LaRue et al, 2004; Zile, 2004); regulation of heart asymmetry (Zile et al., 2000; Zile, 2010); and during heart inflow tract formation where it appears to act as a negative regulator of N-cadherin and TGFβ2 (Romeih et al., 2009; Ghatpande et al., 2010; Zile, 2010).

To address the molecular mechanisms of the interplay between TGFβ2 and RA, an *in vitro* cell culture model was needed. Such a model would enable propagation of defined subsets of cells, diminishing individual differences among individual developing embryos and facilitating the use of standard methods for evaluation of gene expression and for characterization of interactions among regulatory proteins. The cardiovascular marker gene expression and observation of functional cardiomyocyte formation in chick heart forming region tissue and cell cultures showed that these explants are capable to develop and sustain the differentiation process *in vitro* most efficiently when isolated at 3-5 ss and cultured in endothelial cell media, that promoted endothelial cell development.

Addition of exogenous RA in physiological (10 nM) and excess (100 nM) concentrations down-regulated TGFβ2 mRNA and protein expression; this effect was more pronounced in Endo medium than in DMEM. Since RA dose-dependent down-regulation of TGFβ2 was paralleled at protein and mRNA expression level, we conclude that RA blocks TGFβ2 gene transcription.

The TGFβ2 promoter does not contain any RA response elements (Chang et al., 2002), that are needed for a direct regulation of RA of its RAR/RXR complexes, thus the regulation of TGFβ2 by RA should proceed through an indirect mechanism, involving interplay of the growth factors or endocrine effectors at crossroads of cell signaling and regulatory pathways. TGFβ2 expression is governed by two AP-1 binding sites, two AP-2, two Sp1 and four CRE binding sites located in the promoter of this gene (Noma et al., 1991). It is known that RA/RAR/RXR complex can bind to AP-1 thus antagonizing its activity, that is necessary for induction of TGFβ2 expression (Schule et

al., 1991; Dhandapani et al., 2003). Recent studies of amniotic cell membrane explants reveal that Sp1 and liganded RAR/RXR interact physically preventing the Sp1 factor binding to promoter sequence (Borel et al., 2010). Further analysis of TGFβ2 promoter and DNA binding proteins are needed to gather specific data.

The blocking experiments were done with Ro 41 5253 (Ro), a RA antagonist, which was added to the medium in >1000 fold excess over RA. These culture conditions should mimic complete VAD situation observed in the *in vivo* model. Similar to the *in vivo* VAD model, TGFβ2 expression in Ro-blocked primary cell cultures was up-regulated. Due to the great excess of Ro over RA the maximum level of TGFβ2 up-regulation was achieved already at 0.5 μM concentration of the antagonist, while higher Ro doses proved to be less effective for TGFβ2 mRNA and protein synthesis due to possible unspecific side effects on other gene expression.

In the studies with selected genes, that are regulated at mRNA expression level by the presence of TGFβ2 through conventional SMAD signaling pathway in human cell culture (Verrechia et al., 2001), we observed various responses towards exogenous RA, which is down regulating TGFβ2 in chick embryonic cells.

Fibronectin gene FN1 encodes an ECM protein. FN1 gene expression is down-regulated by RA in VAD quail embryo model (Zhou and Zile, unpublished data), in pre-somatic stage - 1 ss (HH3-7) chick embryo explants (Osmond et al., 1991) and in mouse embryonic fibroblast cells NIH-3T3 (Scita et al., 1996). Unlike these models, FN1 mRNA production increased in presence of RA in *in vitro* avian embryonic cell culture, but RA blocking experiments did not affect the expression of FN1. Undoubtedly, ECM formation *in vivo* ad *in vitro* differs substantially and further studies on RA/FN1 interactions should be conducted either *in vivo* or in 3D culture model. Since FN1 gene promoter does not have RARE sequences (Dean et al., 1987), its RA-dependent regulation is likely indirect and may proceed through pathways other than TGFβ2/SMAD signaling.

Neogenin (NEO) is a multifunctional receptor that is related to neural tube development (Cole et al., 2007). Its expression in cultured chick embryo hfr cells indicates the presence of neuronal cell subpopulation, that may be derived from the early neuronal tube region located underneath the hfr of the embryo and were not dissected out during the preparation of explants used for our hfr cell cultures. In human cells the expression of NEO gene is regulated through TGFβ/ SMAD signaling pathway in a time-delayed manner (Verrecchia et al., 2001). We demonstrated that NEO gene mRNA expression in response to RA in chick primary hfr culture parallels the response of TGFβ2 gene mRNA expression; this effect is best seen in the cells grown in Endo medium. NEO is mostly related with neuronal cell lineage, therefore this response could come from neural cells

within hfr primary cell mixture and is not specifically related to heart morphogenesis. The data from VAD quail embryo sections demonstrate that TGFβ2 is expressed in all cell layers, thus the elevated TGFβ2 levels could affect its target genes within other than cardiovascular cell layers. We suggest that in the cultured chick hfr cells NEO may be regulated by RA through a TGFβ2 and SMAD signaling pathway, but this regulation may not be related to heart morphogenesis. More detailed analysis must be done to draw conclusions, because in VAD quail embryos *in vivo* there are no changes in the levels of pSMAD2/3 protein. This must be unambiguously verified in *in vitro* system.

The mRNA expression of the vascular endothelial growth factor VEGF-A is significantly elevated when exogenous RA is present in chick embryonic hfr cell cultures grown in Endo medium, while blocking of RA does not alter the expression of VEGF-A in these cells demonstrating ambiguous mechanisms of RA actions, e.g. presence and absence. RA can induce VEGF mRNA expression through a mechanism that involves SP1 binding sites in the VEGF gene promoter (Akiyama et al., 2002; Maeno et al., 2002). RA-regulated VEGF expression involving TGFβ signaling is reported also in human embryonic stem cells (Sidell et al., 2010). Our data on RA regulation of VEGF-A gene expression in embryonic hfr cell culture corroborate previous assumptions that the regulation of the endothelium growth factor production by exogenous RA may be the molecular mechanism that ameliorates the decrease in endothelial cell numbers associated with vitamin A deficiency (LaRue et al., 2004). Insensitivity of VEGF-A mRNA production to the blocking of RA by its antagonist indicates that RA is not the ultimate regulator of the basal level production of this growth factor, although it may exert considerable regulatory effect, at least at specific stages of the development.

We have developed an *in vitro* model, which can be used to understand the molecular basis of the observed phenomena of TGFβ2 down-regulation in presence of RA. In primary cell cultures from the heart forming regions of 4-7 ss chick embryos, RA supplemented in 10 nM and 100 nM concentration, (i) induces responses in the expression of global regulators TGFβ2 (down-regulated) and HoxBI (up-regulated) opposite to those observed *in vivo* (TGFβ2 up-regulated and HoxB1 being down-regulated in absence of vitamin A) (Maden et al., 1996; Ghatpande et al., 2010) (ii) influences the expression of certain genes, that are regulated by TGFβ2 through SMAD signaling pathway in human cells, although not always in the same way as it is anticipated either from *in vivo* VAD models or considering other *in vitro* models and from the down regulation of TGFβ2 in our hfr cell culture; (iii) induces expression of growth factor VEGF-A mRNA, which is one of the key factors for differentiation of vascular endothelium; (iv) Ro 41 5253, an antagonist of

RA, induces up-regulation of the TGFβ2 and NEO genes, that are down regulated by RA, but exerts no statistically significant influence on the expression of FN1 and VEGF-A genes, that are up-regulated by RA. Research at the molecular level is required to elucidate the mechanism.

In summary, a cell culture model of chick embryo 3-5 ss primary heart-forming region (hfr) cells was developed in Endo media in which it was determined that that this model mimics the effect of the negative regulation of TGFβ2 mRNA and protein expression by RA observed in the *in vivo* VAD quail embryo model. TGFβ2 down-regulation by RA takes place at the gene transcription level; it is indirect due to the absence of RAREs in TGFβ2 promoter region. The developed primary cell culture model can be used to elucidate molecular mechanisms that lead to the down-regulation of TGFβ2 gene mRNA expression by RA.

4.4. Effect of exogenous RA on human heart MSC, endothelial cells and early cardiovascular progenitor cells

Most of the research in the field of developmental biology has been conducted using animal models for studying different molecular interaction mechanisms. The data from our research in VAD quail embryos (Ghatpande et al., 2010) where RA acts as a negative regulator for TGFβ2, is similar to the data from mouse embryogenesis (Mahmood et al., 1992; 1995). Furthermore, our present *in vitro* avian cell culture data of TGFβ2 response to RA are similar to those observed in the avian *in vivo model.*

Here we present the results obtained from *in vitro* cultures of human heart mesenchymal stem cells (MSC), umbilical vein endothelial cells (HUVEC) and early cardiovascular progenitor cells (CPC).

Elevated TGFβ2 protein and gene expression in human heart MSCs in presence of excess RA (66 nM and 133 nM) is not in line with the results chicken hfr cells cultured for 3 days, where in the presence of excess RA (100 nM), the levels of total TGFβ2 are significantly decreased. This discrepancy may be because the developmental stages of the cells are not similar, since the human cells are from adult heart, whereas chicken hfr cells are derived from embryonic explants. The data from 3- and 7-day cultures of 42-day chicken heart cells in presence of excess RA (66 nM and 133 nM) clearly demonstrated a difference between species in TGFβ2 response to the same doses of exogenous RA, leading to preliminary suggestion that RA-regulation of TGFβ2 differs from species to species and is cell-type specific. We suggest that RA regulation of TGFβ2 is at the

transcriptional level since also in human heart MSCs TGFβ2 gene expression in the presence of excess RA mimics that of its total protein level changes in excess RA.

The expression of six of TGFβ/SMAD regulated genes (Verrecchia et al., 2001) was screened in all five human heart MSC cultures but it was difficult to determine the effects of RA on gene expression due to the high standard deviations. The heart MSCs were from five different donors that could be an explanation of individual differences among cell samples. While TGFβ2 gene expression is increased in presence of RA, the expression of FN1 was slightly down-regulated. The data of FN1 correlate with results from avian hfr experiments where in the presence of excess RA the changes of FN1 expression levels are opposite to TGFβ2 expression levels. According to published data, the TGFβ/SMAD regulated genes should respond similarly to the presence of RA. However, this was not the case in the gene expression changes observed here, as most of the genes were either insignificantly down-regulated or not altered after 3- and 7-day culturing. By day 14, the expression of all genes tested was decreased which is not consistent with the TGFβ/SMAD regulation reported previously (Verrecchia et al., 2001). One explanation could be that there is a cell type-specific TGFβ signaling, since the data of target genes come from human fibroblast cell cultures. We must conclude that due to the high variability among heart MSC donor cells, it is difficult to interpret these data; a greater number of samples is required to obtain statistically meaningful data.

HUVEC cells were chosen to test the interaction between RA and TGFβ2 in cells from earlier developmental stages. In the MSCs cells from ~~the~~ human adult heart (Fig.3.14 A), TGFβ2 protein was elevated in the presence of RA at day 3 of culturing, while in the HUVEC cells (Fig. 3.16), the increase in TGFβ2 protein was observed at longer incubation times and the data were not consistent. The differences between HUVEC and hMSC results can be explained by cell type differences. The mean average amount of total TGFβ2 protein from all cultured samples in human MSC control media (2468.7 pg/ml) is similar to HUVEC (2705.7 pg/ml) samples from all time points tested. Previous report from Cenni et al. (2001) shows that RA has no effect on expression levels of TGFβ1 in HUVEC. Our preliminary data suggest that that RA regulation of TGFβ2 is similar in adult heart MSCs and human umbilical vein endothelial cells. However, more research on more HUVEC lines is needed to substantiate this hypothesis.

Elevation of total TGFβ2 protein in human cardiovascular progenitor cells was observed using ELISA kit containing recombinant human TGFβ2. This elevation, however, seems to come from the mouse embryonic fibroblasts (MEFs) that are used as feeder layer for embryonic stem cells and CPCs. The total TGFβ2 protein levels are almost the same in media from MEFs cultured

alone as in media samples taken from CPC cultured on MEFs, suggesting a MEF response to RA, but not CPCs. The primers for TGFβ2 gene analysis were human-specific, since no transcripts were observed in MEF samples. Expression of TGFβ2 is decreased by almost 80% (Fig.3.18) in presence of smaller RA concentration (10 nM) whereas excess RA (100 nM) leads to 35% decrease in TGFβ2 transcript synthesis, leading to suggestion that these cells are more sensitive to physiological and excess levels of RA Interestingly, the previously reported (Verrecchia et al., 2001) TGFβ/SMAD target gene, FN1, responds similarly to the presence of RA. This response differs from our avian primary cell *in vitro* data. However, the observed changes of TGFβ2 gene expression in presence of RA at physiological levels are in line with the data from avian hfr studies obtained from *in vivo* VAD quail embryos, suggesting that RA-regulated mechanisms are conserved among species during early embryonic development. However, the data are insufficient and more cell lines need to be tested to confirm these observations. These data also suggest the CPCs as potential human cell model for further research of the development of the cardiovascular system *in vitro*.

In summary, we have made the following observations: that RA regulation of TGFβ2: (i) in adult heart cells differs between species; (ii) is similar in adult heart MSCs and human umbilical vein endothelial cells; (iii) is cell type-specific and (iv) that TGFβ2 regulation by RA is likely conserved among species during vertebrate early embryonic development.

Understanding how networks of regulatory signals work during cardiovascular development will help to understand the etiology of congenital cardiovascular malformations, which are the major birth defects (August and Suthanathiran, 2006).

The observed interactions between TGFβ2 and RA could be tested on several disease models that involve elevated TGFβ2 levels. One of such is Alzheimers disease (AD). Noguchi et al. (2010) discovered that an upregulation of the TGFβ2 level is a common pathological feature of AD brains; they suggest that elevated TGFβ2 may be closely linked to the–neuronal death related to AD. However, the regulatory mechanisms in absence and in excess RA are different therefore this interplay must be investigated in the involved cell types, since many studies in the field of cancer demonstrate the role of excess RA as an inhibitor of malignant cell growth through a positive regulation of TGFβ2 (Jakowlew et al., 2000; Singh et al., 2007)

5. Conclusions

The vitamin A active form at the gene level is retinoic acid (RA), a strong morphogen influencing vertebrate development, including embryogenesis. Deficiency of vitamin A during embryogenesis manifests in abnormalities of cardiovascular development that have been demonstrated in several species. However, the function of vitamin A at very early stages of heart development have not been studied. In this work we have examined the interactions between RA and the global growth factor TGFβ2 during early heart morphogenesis. We have made the following novel observations:

1. The vitamin A-deficient *in vivo* model (VAD quail embryo) demonstrates abnormal morphological and biochemical changes during heart development, including the closure of cardiac inflow tracts, and elevated mRNA levels of N-cadherin and TGFβ2. TGFβ2 transcripts are localized in the heart forming regions of normal and VAD embryos.
2. Administration of TGFβ2 anti-sense to the VAD embryo results in the normalization of inflow tract development suggesting that TGFβ2 is involved in IFT morphogenesis. This hypothesis is supported by data obtained from normal quail embryos injected with TGFβ2 active protein, that leads to VAD-like abnormalities in early heart development.
3. In our laboratory in vitro cell culture system has been developed from chicken heart forming region cells which can be used as a base for further detailed mechanistic studies of interaction between RA and TGFβ2.
4. The expression level of HoxBI *in vitro* is elevated when RA is administered in both concentrations verifying the functionality of RA signaling in the primary cell culture.
5. Importantly, our studies determined that RA acts as a negative regulator of TGFβ2 in cardiovascular cells *in vivo* and *in vitro*, validating the use of the cell culture model for further studies of the RA/ TGFβ2 interaction.
6. The target genes of TGFβ/SMAD pathway, Fibronectin 1 (FN1) and neogenin (NEO), were found to be altered both in the presence of excess RA and when blocking RA signaling *in vitro*. In the presence of excess RA, expression of FN1 is elevated, but blocking of RA signaling does not change FN1 expression. NEO is down-regulated in the presence of excess RA, while blocking RA signaling leads to a strong up-regulation of NEO expression.
7. The data of increased levels of VEGF transcripts in the presence of RA in our *in vitro* system correspond to the morphological and histological observations from VAD quail embryos *in vivo*.

8. Cell cultures from human heart mesenchymal stem cells and from human umbilical vein endothelial cells demonstrated a different response in TGFβ2 mRNA expression and protein accumulation in response to RA compared to that observed in avian cell cultures, where there was a decrease of TGFβ2 mRNA and protein in the presence of exogenous RA.
9. Preliminary results from human embryonic stem cell derived cardiac progenitor cell line (CPC) suggest that these cells can be used as a model system for further studies on RA and its regulated molecules since their responses were similar to whose obtained in avian hfr *in vitro* system.

6. Defense thesis

1. During early heart development (4-5 ss) the absence of vitamin A in the *in vivo* VAD quail embryo leads to an increased accumulation of TGFβ2 protein (a ligand for many signaling pathways) and its mRNA in the heart forming regions and subsequently in the heart inflow tracts during heart morphogenesis. Strict regulation of TGFβ2 gene transcription by retinoic acid at this stage is necessary for normal heart development.

2. The effects of RA observed *in vivo* can be duplicated in the *in vitro* systems of avian heart forming cells and human early cardiovascular progenitor cells.

3. The interaction of TGFβ2 and retinoic acid is time and cell type dependent. Further studies on molecular mechanisms and species-specific features can be conducted in either avian or human heart forming cells in culture.

4. The *in vitro* data of the effect of RA on VEGF and *in vivo* observations from VAD quail embryos, together with data from literature showing lower numbers of endothelial cells in VAD embryos suggest that RA is involved in the regulation of endothelial cells during early cardiovascular development.

7. Acknowledgements and funding

These studies were supported by NIH grant 5R01 HL61982-03 (to M.H.Zile), National Research Initiative of the USDA Cooperative State Research, Education and Extension Service Grants 2000-35200-9062 and 2005-35200-15257 (to M.H.Zile), by the Michigan Agricultural Experiment Station, the European Social Fund (ESF) and European Regional Development Fund (ERDF), Latvian Science Council, L'ORÉAL Latvija and University of Latvia.

I wish to express my sincere gratitude towards Prof. Indriķis Muižnieks for introducing me to the field of molecular microbiology and molecular biology and for his continuous support throughout the time of my studies and work at the University of Latvia, Department of Microbiology and Biotechnology.

I am very grateful to Prof. Maija H.Zile for introducing me to the field of developmental biology and guiding me through the PhD work and for very constructive critic and discussions. I can never stop learning from you.

Many thanks are due to Dr. Martin Hoogduijn, Erasmus Medical Center and Dr. Michele Pucéat and his group from INSERM laboratories, for cell material and pleasant experience in your laboratories.

My gratitude to wonderful colleagues in MSU, Nicolette Brown, Kathy Barret, Elizabeth Rondini, Jian Cui, Helen Dersch and Mahmood Romeih.

I am sincerely thankful to Dr. Janis Ancans, Dr. Una Riekstina, Prof. Ruta Muceniece Dr. Ilva Nakurte and my colleagues Anna Ramata-Stunda, Janis Liepins, Valdis Pirsko, Reinis Rutkis and Anete Keisa for all the scientific discussions.

I would like to thank all the friends and colleagues who have been with me.

Last, but not least, I want to thank my family for their support and understanding.

8. References

Abu-Issa, R, Waldo, K. and Kirby, M.L. 2004. Heart fields: ones, two or more? Dev Biol. 272:281-285.

Abu-Issa, R. and Kirby, M.L. 2008. Patterning of the heart field in the chick. Dev Biol. 319; 223-233.

Adams, A., Thorn, J.M., Yamabhai, M., Kay, B.K. and O'Bryan, J.P. 2000. Intersectin, and adaptor protein involved in clathrin-mediated endocytosis, activates mitogenic signaling pathways. J Biol Chem. 275:27414-27420.

Andres, J.L., Stanley, K., Cheifetz, S. and Massagué, J. 1989. Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-beta. J Cell Biol. 109:3137-3145.

Apfel, C., Bauer, F., Crettaz, M., Forni, L., Kamber, M., Kaufmann, F., LeMotte, P., Pirson, W. and Klaus, M. 1992. A retinoic acid receptor α antagonist selectively counteracts retinoic acid effects. Proc Natl Acad Sci USA. 89:7129-7133.

Attisano, L. and Labbé, E. 2004. TGFβ and Wnt pathway cross-talk. Cancer Metastasis Rev. 23:53-61.

Austin, A.F., Compton, L.A., Love, J.D., Brown, C.B. and Barnett, J.V. 2008. Primary and immortalized mouse epicardial cells undergo differentiation in response to TGFβ. Dev Dyn. 237:366-376.

Azhar, M., Runyan, R.B., Gard, C., Sanford, L.P., Miller, M.L., Andringa, A., Pawlowski, S., Rajan, S. and Doetschman, T. 2009. Ligand-specific function of transforming growth factor beta in epithelial-mesenchymal transition in heart development. Dev Dyn. 238: 431-442.

Azhar, M., Schultz ,J.E.J., Grupp, I., Dorn II, G.W., Meneton, P., Molin, D.G.M., Gittenberger-de Groot, A.C. and Doetschman, T. 2003. Transforming growth factor beta in cardiovascular development and function. Cytokine & Growth Factor Revs. 14:391-407.

Balmer, J.E. and Blomhoff, R. 2002. Gene expression regulation by retinoic acid. J Lipid Res. 43:1773-1808.

Balmer, J.E. and Blomhoff, R. 2005. A robust characterization of retinoic acid response elements based on a comparison of sites in three species. J Steroid Biochem Mol Biol. 96:347-354.

Bartholin, L., Powers, S.E., Melhuish, T.A., Lasse, S., Weinstein, M. and Wotton, D. 2006. TIGF inhibits retinoid signaling. Mol Cell Biol. 26:990-1001.

Barua, A.B. and Sidell, N. 2004. Retinol beta-glucuronide: a biologically active interesting retinoid. J Nutr. 134:286S-289S.

Batten, M.L., Imanishi, Y., Maeda, T., Tu, D.C., Moise, A.R., Bronson, D., Possin, D., Van Gelder, R.N., Baehr, W. and Palczewski, K. 2004. Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver. J Biol Chem. 279:10422-10432.

Benchabane, H. and Wrana, J.L. 2003. GATA- and Smad1-dependent enhancers in the Smad7 gene differentially interpret bone morphogenetic protein concentrations. Mol Cell Biol. 18: 6646– 6661.

Black, B.L. 2007. Transcriptional pathways in second heart field development. Semin Cell Dev Biol. 18:67-76.

Blin, G., Nury, D., Stefanovic, S., Neri, T., Guillevic, O., Brinon, B., Bellamy, V., Rücker-Martin, C., Barbry, P., Bel, A., Bruneval, P., Cowan, C., Pouly, J., Mitalipov, S., Gouadon, E., Binder, P., Hagège, A., Desnons, M., Renaud, J.F., Menasché, P. and Pucéat, M. 2010. A purified population of multipotent cardiovascular progenitors derived from primate pluripotent stem cells engrafts in postmyocardial infarcted nonhuman primates. J Clin Invest. 120:1125-1139.

Blomhoff, R. 1997. Retinoids may increase fibrotic potential of TGF-β: crosstalk between two multifunctional effectors. Hepatology 22:1067-1078.

Blomhoff, R. and Blomhoff, H.K. 2006. Overview of retinoid metabolism and function. J Neurobiol. 66:606-630.

Blomhoff, R., Green, M.H., Berg, T. and Norum, K.R. 1990. Transport and storage of vitamin A. Science 250:399-404.

Bobik, A. 2006. Transforming growth factor-βs and vascular disorders. Arterioscler Thromb Vasc Biol. 26:1712-1720.

Boettger, T., Wittler, L. M. and Kessel, M. 1999. FGF8 functions in the specification of the right body side of the chick. Curr Biol. 9:277–280.

Bohnsack, B.L. and Hirschi, K.K. 2004. Red light, green light. Signals that control endothelial cell proliferation during embryonic vascular development. Cell Cycle 3:1506-1511.

Boncinelli, E., Simeone, A., Acampora, D. and Mavilio, F. 1991. HOX gene activation by retinoic acid. Trends Genet. 7:329-334.

Borel, V., Marceau, G., Gallot, D., Blanchon, L. and Sapin, V. 2010. Retinoids regulate human amniotic tissue-type plasminogen activator gene by a two-step mechanism. J Cell Mol Med. 14:1793-1805.

Böttner, M., Krieglstein, K. and Unsicker, K. 2000. The transforming growth factor-βs: structure, signaling, and roles in neuronal development and functions. J Neurochem. 75:2227-2240.

Bouwman, P. and Philipsen, S. 2002. Regulation of the activity of Sp1-related transcription factors. Mol Cell Endocrinol. 195:27-38.

Brand, T. 2003. Heart development: molecular insights into cardiac specification and early morphogenesis. Dev Biol. 258:1-19.

Burt, D.W., Paton, I.R. and Dey, B.R. 1991. Comparative analysis of human and chicken transforming growth factor-beta 2 and -beta 3 promoters. J Mol Endocrinol. 7:175-183.

Butts, S.C., Liu, W., Li, G. and Frenz, D.A. 2005. Transforming growth factor-β1 signaling participates in the physiological and pathological regulation of mouse inner ear development by all-trans retinoic acid. Birth Defects Res. 73:218–228.

Buxbaum, J.N. and Reixach, N. 2009. Transthyretin: the servant of many masters. Cell Mol Life Sci. 66:3095-3101.

Campo-Paysaa, F., Marlétaz, F., Laudet, V. and Schubert, M. 2008. Retinoic acid signaling in development: tissue-specific functions and evolutionary origins. Genesis. 46:640-656.

Cenni, E., Granchi, D., Ciapetti, G., Savarino, L., Vancini, M. and Leo, A.D. 2001. Effect of CMW 1 bone cement on transforming growth factor-beta 1 expression by endothelial cells. J Biomater Sci Polym Ed. 12:1011-1025.

Chan-Thomas, P.S., Thompson, R.P., Robert, B., Yacoub, M.H. and Barton, P.J. 1993. Expression of homeobox genes Msx-1 (Hox-7) and Msx-2 (Hox-8) during cardiac development in the chick. Dev Dyn. 197:203-216.

Chang, H., Brown, C.W. and Matzuk, M.M. 2002. Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev. 23:787-823.

Chang, L. and Karin, M. 2001. Mammalian MAP kinase signaling cascades. Nature 410:37-40.

Charng, M.J., Frenkel, P.A., Lin, Q., Yamada, M., Schwartz, R.J., Olson, E.N., Overbeek, P., Schneider, M.D. and Yumada, M. 1998. A constitutive mutation of ALK5 disrupts cardiac looping and morphogenesis in mice. Dev Biol. 199:72–79.

Cheifetz, S., Bellón, T., Calés, C., Vera, S., Bernabeu, C., Massagué, J. and Letarte, M. 1992. Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J Biol Chem. 267:19027-19030.

Chen, F., Desai, T.J., Qian, J., Niederreither, K., Lu, J. and Dardoso, W.V. 2007. Inhibition of Tgf β signaling by endogenous retinoic acid is essential for primary lung bud induction. Development 134:2969-2979.

Chen, H. and Juchau, M.R. 1998. Biotransformation of 13-cis- and 9-cis-retinoic acid to all-trans-retinoic acid in rat conceptal homogenates. Evidence for catalysis by conceptal isomerase. Drug Metab Dispos. 26:222-228.

Chen, Y., Kostetskii, I., Zile, M.H. and Solursh, M. 1995. Comparative study of Msx-1 expression in early normal and vitaminA-deficient avian embryos. J Exp Zool. 272:299-310.

Chen, Y.H., Ishii, M., Sun, J., Sucov, H.M. and Maxon, R.E.Jr. 2007. Msx1 and Msx2 regulate survival of secondary heart field precursors and post-migratory proliferation of cardiac neural crest in the outflow tract. Dev Biol. 308:421-437.

Cheng, Y.H., Yin, P., Xue, Q., Yilmaz, B., Dawson, M.I. and Bulun, S.E. 2008. Retinoic acid (RA) regulates 17β-hydroxysteroid dehydrogenase type 2 expression in endometrium: interactions of RA receptors with specificity protein (SP) 1/SP3 for estradiol metabolism. J Clin Endocrinol Metab. 93:1915-1923.

Christoffels, V.M., Burch, J.B.E. and Moorman, A.F.M. 2004. Architecture plan for the heart: early patterning and delineation of the chambers and the nodes. Trends Cardiovasc Med. 14:301-307.

Coberly, S., Lammer, E. and Alashari, M. 1996. Retinoic acid embryopathy: case repot and review of literature. Pediatr Pathol Lab Med. 16:823-836.

Colakoglu, N., Kukner, A., Oner, J., Sonmez, M.F., Oner, H. and Ozan, E. 2006. Effects of high dose retinoic acid on TGF-β2 expression during pancreatic organogenesis. J Mol Histol. 36:413–418.

Cole, S.J., Bradford, D. and Cooper, H.M. 2007. Neogenin: a multi-functional receptor regulating diverse developmental processes. Int J Biochem Cell Biol. 39:1569-1575.

Collins, M.D. and Mao, G.E. 1999. Teratology of retinoids. Annu rev Pharmacol Toxicol. 39:3999-430.

Crews, H., Alink, G., Andersen, R., Braesco, V., Holst, B., Mainani, G., Ovesen, L., Scotter, M., Solfrizzo, M., van den Berg, R., Verhagen, H. and Williamson, G. 2001. A critical

assessment of some biomarker approaches linked with dietary intake. Br J Nutr. 86 Suppl 1:S5-35.

Cui, C., Cheuvront, T.J., Lansford, R.D., Moreno-Rodriguez, R.A., Schultheiss, T.M. and Rongish, B.J. 2009. Dynamic positional fate map of the primary heart-forming region. Dev Biol. 332:212-222.

Cui, J., Michaille, J.J., Weihong, J. and Zile, M.H. 2003. Retinoid receptors and vitamin A deficiency: differential patterns of transcription during early avian development and the rapid induction of RARs by retinoic acid. Dev Biol. 260:496-511.

Cui, W., Fowlis, D.J., Brysons, S., Duffie, E., Ireland, H., Balmain, A. and Akhurstm, R. J. 1996. TGFb1 inhibits the formation of benign skin tumors, but enhances the progression to invasive spindle carcinomas in transgenic mice. Cell 86:531-542.

Cullingworth, J., Hooper, M.L., Harrison, D.J., Mason, J.O., Sirard, C., Patek, C.E. and Clarke, A.R. 2002. Carciongen-induced pancreatic lesions in the mouse: Effect of Smad4 and Apc genotypes. Oncogene 21:4696-4701.

Daopin, S., Piez, K.A., Ogawa, Y. and Davies, D.R. 1992. Crystal structure of transforming growth factor-beta 2: an unusual fold for the superfamily. Science 257:369-373.

Davidson, L.A., Marsden, M., Keller, R. and Desimone, D.W. 2006. Integrin alpha5beta1 and fibronectin regulate polarized cell protrusions required for Xenopus convergence and extension. Curr. Biol. 16:833-844.

de Lera, A.R., Bourguet, W., Altucci, L. and Gronemeyer, H. 2007. Design of selective nuclear receptor modulators: RAR and RXR as a case study. Nat Rev Drug Discov. 6:811-820.

De Luca, L.M. 1991. Retinoids and their receptors in differentiation, embryogenesis and neoplasia. FASEB J. 5:2924-2933.

Dean, D.C., Bowlus, C.L. and Bourgeois, S. 1987. Cloning and analysis of the promoter region of the human fibronectin gene. Proc Natl Acad Sci USA 84:1876-1880.

Degos, L. and Wang, Z.Y. 2001. All trans retinoic acid in acute promyelocytic leukemia. Oncogene 20:7140-7145.

del Re, E., Babitt, J.L., Pirani, A., Schneyer, A.L. and Lin, H.Y. 2004. In the absence of type III receptor, the transforming growth factor (TGF)-beta type II-B receptor requires the type I receptor to bind TGF-beta2. J Biol Chem. 279:22765-22772.

Dersch, H. and Zile, M.H. 1993. Induction of normal cardiovascular development in the vitamin A-deprived quail embryo by natural retinoids. Dev Biol.16:424-433.

Derynck, R. and Zhang, Y.E. 2003. Smad-dependent and Smad-independent pathways in TGF-β family signalling. Nature 425:577-584.

Derynck, R., Gelbart, W.M., Harland, R.M., Heldin, C.H., Kern, S.E., Massagué, J., Melton, D.A., Mlodzik, M., Padgett, R.W., Roberts, A.B., Smith, J., Thomsen, G.H., Vogelstein, B. and Wang, X.F. 1996. Nomenclature: vertebrate mediators of TGFbeta family signals. Cell 87:173.

Dhandapani, K.M., Hadman, M., De Sevilla, L., Wade, M.F., Mahesh, V.B. and Brann, D.W. Astrocyte protection of neurons. Role of transforming growth factor-β signaling via c-Jun-FAP-1 protective pathway. J Biol Chem. 278:43329-43339.

Dickson, M.C., Lager, H.G., Duffie, E., Mummery, C.L. and Akhurst R.J. 1993. RNA and protein localizations of TGFβ2 in the early mouse embryo suggest an involvement in cardiac development. Development 117:625-639.

Dickson, M.C., Martin, J.S., Cousins, F.M., Kulkarni, A.B., Karlsson, S. and Akhurst, R.J. 1995. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development 121:1845-1854.

Dong, D. and Zile, M.H. 1995. Endogenous retinoids in the early avian embryo. Biochem Biophys Res Commun. 217:1026-1031.

Dong, D., Ruuska, S.E., Levinthal, D.J. and Noy, N. 1999. Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. J Biol Chem. 274:23695-23698.

Duester, G., Mic, F.A. and Molotkov, A. 2003. Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid. Chem Biol Interact. 1:143-144:201-210.

Dünker, N. and Krieglstein, K. 2000. Targeted mutations of transforming growth factor-β genes reveal important roles in mouse development and adult homeostasis. Eur J Biochem. 267:6982-6988.

Dupé, V. and Lumsden, A. 2001. Hindbrain patterning involves graded responses to retinoic acid signalling. Development, 128:2199-2208.

Dupé, V., Matt, N., Garnier, J.M., Chambon, P. Mark, M. and Ghyselinck, N.B. 2003. A newborn lethal defect due to inactivation of retinaldehyde dehydrogenase type 3 is prevented by maternal retinoid acid treatment. Proc Natl Acad Sci USA. 100:14036-14041.

Durand, B., Saunders, M., Leroy, P., Leid, M. and Chambon, P. 1992. All-trans and 9-cis retinoic acid induction of CRABPII transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. Cell 71:73-85.

Eckhoff, C. and Nau, H. 1990. Identification and quantitation of all-trans- and 13-cis-retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. J Lipid Res. 31:1445-1454.

Eichmann, A., Yuan, L., Moyon, D., Lenoble, F., Pardanaud, L. and Breant, C. 2005. Vascular development: from precursor cells to branched arterial and venous networks. Int J Dev Biol. 49:259-267.

Esparza-López, J., Montiel, J.L., Vilchis-Landeros, M.M., Okadome, T., Miyazono, K. and López-Casillas, F. 2001. Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor-beta superfamily. Specialized binding regions for transforming growth factor-beta and inhibin A. J Biol Chem. 276:14588-14596.

Euler-Taimor, G. and Heger, J. 2006. The complex pattern of SMAD signaling in the cardiovascular system. Cardiovascular Res. 69:15-25.

Feng, X.H. and Derynck, R. 2005. Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol. 21:659-693.

Ferguson, M.W.J. and O'Kane, S. 2004. Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. Phil Trans R Soc Lond. 359:839-850.

Ferrara, N., Houck, K.A., Jakeman, L.B., Winer, J. and Leung, D.W. 1991. The vascular endothelial growth factor family of polypeptides. J Cell Biochem. 47:211-218.

Ferrara, N. 2000. Vascular endothelial growth factor and the regulation of angiogenesis. Recent Prog Horm Res. 55:15-36.

Fishman, M.C. and Chien, K.R. 1997. Fashioning the vertebrate heart: earliest embryonic decisions. Development 124:2099-2117.

Fong, G.H., Zhang, L., Bryce, D.M. and Peng, J. 1999. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development 126:3015-3025.

Fraser, P.D. and Bramley, P.M. 2004. The biosynthesis and nutritional uses of carotenoids. Prog Lipid res. 43:228-65.

Freitas, C.S. and Dalmau, S.R. 2006. Multiple sources of non-embryonic multipotent stem cells: processed lipoaspirates and dermis as promising alternatives to bone-marrow-derived cell therapies. Cell Tissue Res. 325:403-411.

Funaba, M., Zimmerman, C.M. and Mathews, L.S. 2002. Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. J Biol Chem. 277:41361-8.

Galiher, A.J. and Schiemann, W.P. 2007. Src phosphorylates Tyr284 in TGF-beta type II receptor and regulates TGF-beta stimulation of p38 MAPK during breast cancer cell proliferation and invasion. Cancer res. 67:3752-3758.

Gaussin, V., Van de Putte, T., Mishina, Y., Hanks, M.C., Zwijsen, A., Huylebroeck, D., Behringer, R.R. and Schneider, M.D. 2002. Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3. Proc Natl Acad Sci USA. 99:2878-2883.

George, E.L., Georges-Labouesse, E.N., Patel-King, R.S., Rayburn, H. and Hynes, R.O. 1993. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. Development 119:1079-1091.

Germain, P., Chambon, P., Eichele, G., Evans, R.M., Lazar, M.A., Leid, M., de Lera, A.R., Lotan, R., Mangelsdorf, D.J. and Gronemeyer, H. 2006a. International union of pharmacology.LX. Retinoic acid receptors. Pharmacol Rev. 58:712-725.

Germain, P., Chambon, P., Eichele, G., Evans, R.M., Lazar, M.A., Leid, M., de Lera, A.R., Lotan, R., Mangelsdorf, D.J. and Gronemeyer, H. 2006b. International union of pharmacology.LXIII. Retinoid X receptors. Pharmacol Rev. 58:760-772.

Ghatpande, S.K. 2008. Gallera method of chick embryo culture in vitro supports better growth compared with original New method. Dev Growth Differ. 50:437-442.

Ghatpande, S.K., Zhou, H.R., Cakstina, I., Carlson, C., Rondini, E.A., Romeih, M. and Zile, M.H. 2010. Transforming growth factor beta2 is negatively regulated by endogenous retinoic acid during early heart morphogenesis. Dev Growth Differ. 52:433-455.

Giguere, V., Ong, E.S., Segui, P. and Evans, R.M. 1987. Identification of a receptor for the morphogenic retinoic acid. Nature 330:624-629.

Glick, A.B., Flanders, K.C., Danielpour, D., Yuspa, S.H. and Sporn, M.B. 1989. Retinoic acid induces transforming growth factor-beta 2 in cultured keratinocytes and mouse epidermis. Cell Regul. 1:87-97.

Glick, A.B., McCune, B.K., Abdulkarem, N., Flanders, K.C., Lumadue, J.A., Smith, J.M. and Sporn, M.B. 1991. Complex regulation of TGFβ expression by retinoic acid in the vitamin A-deficient rat. Development 111:1081-1086.

Goetz, S. and Conlon, F.L. 2007. Cardiac progenitors and the embryonic cell cycle. Cell Cycle 6:1974-1981.

Gordon, K.J. and Blobe, G.C. 2008. Role of transforming growth factor-β superfamily signaling pathway in human disease. Biochim Biophys Acta 1782:197-228.

Goumans, M.J. and Mummery, C. 2000. Functional analysis of the TGFβreceptor/Smad pathway through gene ablation in mice. Int. J. Dev.Biol. 44:253-265.

Goumans, M.J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P. and ten Dijke P. 2002. Balancing the activation state of the endothelium via two distinct TGF-β type I receptors. EMBO J. 21:1743-1753.

Govinden, R. and Bhoola, K.D. 2003. Genealogy, expression, and cellular function of transforming growth factor-beta. Pharmacol Ther. 98:257-265.

Grépin, C., Nemer, G. and Nemer, M. 1997. Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. Development 12:2387-2395.

Gressner, O.A., Weiskirchen, R. and Gressner, A.M. 2007. Evolving concepts of liver fibrogenesis provide new diagnostic and therapeutic options. Comp Hepatol. 30:6-7.

Griendling, K.K., Harrison, D.G. and Alexander, R.W. 2000. Molecular and cellular biology of blood vessels. Hurst's The Heart, Vol2, 10th Edition, Chapter 6.

Guo, X. and Wang, X.F. 2009. Signaling cross-talk between TGF-beta/BMP and other pathways. Cell Res. 19:71-88.

Hamade, A., Deries, M., Begemann, G., Bally-Cuif, L., Geneˆt, C., Sabatier, F., Bonnieu, A. and Cousin, X. 2006. Retinoic acid activates myogenesis in vivo through FGF8 signaling. Dev Biol. 289:127-140.

Hamburger, V. and Hamilton, H.L. 1951. A series of normal stages in the development of the chick embryo. J Morphol. 88:49-92. Reprint in Dev Dyn 1992.195:231-272.

Harrison, E.H. 2005. Mechanisms of digestion and absorption of dietary vitamin A. Annu Rev Nutr. 25:87-103.

Harvey, R. 2002. Patterning the vertebrate heart. Nature Reviews Genetics 3:544-556.

Hatta, K., Takagi, S., Hajime, F. & Takeichi, M. 1987. Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryo. Dev Biol. 120:215-227.

Hay, E.D. 1981. Extracellular matrix. J Cell Biol. 91:205s-223s.

Hay, E.D. 2005. The mesenchymal cell, its role in the embryo, and remarkable signaling mechanisms that create it. Dev Dyn. 233:706-720.

Heine, U.I., Roberts, A.B., Munoz, E.F., Roche, N.S. and Sporn, M.B. 1985. Effects of retinoid deficiency on the development of the heart and vascular system of the quail embryo. Virchows Arch B Cell Pathol Incl Mol Pathol. 50:135-52.

Heldin, C.H., Miyazono, K. and ten Dijke, P. 1997. TGF-beta signalling form the cell membrane to nucleus through SMAD proteins. Nature 390:465-471.

Hernandez, R.E., Putzke, A.P., Myers, J.P., Margaretha, L. and Moens, C.B. 2007. Cyp26 enzymes generate the retinoic acid response pattern necessary for hindbrain development. Development 134:177-187.

Herr, F.M., Wardlaw, S.A., Kakkad, A., Albrecht, A., Quick, T.C. and Ong, D.E. 1993. Intestinal vitamin A metabolism: coordinate distribution of enzymes and CRBP(II). J Lipid Res. 34:1545-1554.

Hirosawa, K. and Yamada, E. 1973. The localization of the vitamin A in the mouse liver as revealed by electron microscope radioautography. J Electron Microscopy. 22:337-346.

Hoffman, L.M., Weston, A.D. and Underhill, T.M. 2003. Molecular mechanisms regulating chondroblast differentiation. J Bone Joint Surg Am. 85-A Suppl 2:124-32.

Hogan, B.L. 1996. Bone morphogenic proteins: multifunctional regulators of vertebrate development. Genes Dev. 10:1580-1594.

Hoover, L.L., Burton, E.G., Brooks, B.A. and Kubalak, S.W. 2008. The expanding role for retinoid signaling in heart development. Scientific world journal 8:194-211.

Hoover, L.L., Burton, E.G., O'Neil, M.L., Brooks, B.A., Sreedharan, S., Dawson, N.A. and Kubalak, S.W. 2008. Retinoids regulate TGFbeta signaling at the level of Smad2 phosphorylation and nuclear accumulation. Biochim Biophys Acta. 1783:2279-2286.

Howat,W.J., Holgate, S.T. and Lackie, P.M. 2002. TGF-β isoform release and activation during in vitro bronchial epithelial wound repair. Am J Physiol Lung Cell Mol Physiol.282:L115-L12.

Huelsken, J., Vogel, R., Brinkmann, B., Birchmeier, C. and Birchmeier, W. 2000. Requirement for beta-catenin in anterior-posterior axis formation in mice. J Cell Biol. 148:567-578.

Hyytiäinen, M., Taipale, J., Heldin, C.H. and Keski-Oja, J. 1998. Recombinant latent transforming growth factor-b-binding protein 2 assembles to fibroblast extracellular matrix and is susceptible to proteolytic processing and release. J Biol Chem. 273:20669-20676.

Ide, H. and Aono, H. 1988. Retinoic acid promotes proliferation and chondrogenesis in the distal mesodermal cells of chick limb bud. Dev Biol. 130:767-773.

Inman, G.J., Nicolás, F.J. and Hill, C.S. 2002. Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. Mol Cell 10:283-94.

Iulianella, A. and Lohnes, D. 2002. Chimeric analysis of retinoic acid receptor function during cardiac looping. Dev Biol. 247:62-75.

Jakowlew, S.B., Dillard, P.J., Sporn, M.B. and Roberts, A.B. 1990. Complementary deoxyribonucleic acid cloning of an mRNA encoding transforming growth factor-beta 2 from chicken embryo chondrocytes. Growth Factors 2:123-133.

Jakowlew, S.B., Ciment, G., Tuan, R.S., Sporn, M.B. and Roberts, A.B. 1994. Expression of transforming growth factor-β2 and -β3 mRNAs and proteins in the developing chicken embryo. Differentiation 55:105-118.

Jakowlew, S.B., Zakowicz, H and Moody, T.W. 2000. Retinoic acid down-regulates VPAC(1) receptors and TGF-beta 3 but up-regulates TGF-beta 2 in lung cancer cells. Peptides 21:1831-1837.

Jetten, A. 2009. Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism. Nuclear Receptor Signaling 7:e003.

Jiang, Y., Tarzami, S., Burch, J.B. and Evans, T. 1998. Common role for each of the cGATA-4/5/6 genes in the regulation of cardiac morphogenesis. Dev Genet. 22:263-277.

Jiao, K., Langwworthy, M., Batts, L., Brown, C.B., Moses, H. and Baldwin, H.S. 2006. Tgfβ signaling is required for atrioventricular cushion mesenchyme remodeling during in vivo cardiac development. Development 133:4585-4593.

Kaartinen, V., Voncken, J.W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N. and Groffen, J. 1995. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. Nat Genet. 11:415-421.

Kanzaki, T., Shiina, R., Saito, Y., Oohashi, H. and Morisaki, N. 1998. Role of latent TGF-beta 1 binding protein in vascular remodeling. Biochem Biophys Res Commun. 246:26-30.

Kardassis, D., Murphy, C., Fotsis, T., Moustakas, A. and Stournaras, C. 2009. Control of transforming growth factor beta signal transduction by small GTPases. FEBS J. 276: 2947-2965.

Kastner, P., Mark, M. and Chambon, P. 1995. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? Cell 83:859-869.

Kawaguchi, R., Yu, J., Honda, J., Hu, J., Whitelegge, J., Ping, P., Wiita, P., Bok, D. and Sun, H. 2007. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. Science 315:820-825.

Keller, B.B., Wessels, A., Schwartz, R.J., Roberts, R. and Markwald, R. 2000. Molecular development of the heart. Hurst's The Heart, Vol2, 10th Edition, Chapter 9.

Khali, N. 1999. TGF-beta: from latent to active. Mircobes Infect. 1:1255-1263.

Keidel, S., Lemot1e, P. and Apfel, C. 1994. Different agonist- and antagonist-induced conformational changes in retinoic acid receptors analyzed by protease mapping. Mol Cell Biol. 14:287-298.

Kingsley, D.M. 1994. The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev. 8:133-146.

Koli, K. and Keski-Oja, J. 1993. Vitamin D3 and calcipotriol decrease pericellular plasminogen activator activity in cultured keratinocytes. J Invest Dermatol. 101:706–712.

Koli, K., Saharinen, J., Hyytiäinen, M., Penttinen, C. and Keski-Oja, J. 2001. Latency, activation, and binding proteins of TGF-beta. Micorc Res Tech. 52:354-362.

Kostetskii, I. and Zile, M.H. 1993. Expression of genes for retinoic acid receptors and TGFβ during quail embryogenesis. FASEB J. 522A.

Kostetskii, I., Jiang, Y., Kostetskaia, E., Yuan, S., Evans, T. and Zile M.H. 1999. Retinoid signaling required for normal heart development regulates GATA4 in a pathway distinct from cardiomiocyte differentitation. Dev Biol. 206:206-218.

Kostetskii, I., Li, J., Xiong, Y., Zhou, R., Ferrari, V.A., Patel, V.V., Molkentin, J.D. and Radice, G.L. 2005. Induced deletion of the N-cadherin gene in the heart leads to dissolution of the intercalated disc structure. Circ Res. 96:346-354.

Kostetskii, I., Yuan, S.Y., Kostetskaia, E., Linask, K., Blanchet, S., Seleiro, E., Michaille, J.J., Brickell, P. and Zile, M.H. 1998. Initial retionoid requirement for early avian development concides with retionoid receptor coexpression in the precardiac fields and induction of normal cardiovascular development. Dev Dyn. 213:188-198.

Krug, E.L., Mjaatvedt, C.H. and Markwald, R.R. 1987. Extracellular matrix from embryonic myocardium elicits an early morphogenic event in cardiac endothelial differentiation. Dev Biol. 120: 348-355.

Kubalak, S.W., Hutson, D.R., Scott, K.K. and Shannon, R.A. 2002. Elevated transforming growth factor beta2 enhances apoptosis and contributes to abnormal outflow tract and aortic sac development in retinoic X receptor alpha knockout embryos. Development 129:733-746.

Kulkarni, A.B., Huh, C.G., Becker, D., Geiser, A., Lyght, M., Flanders, K.C., Roberts, A.B., Sporn, M.B., Ward, J.M. and Karlsson, S. 1993. Transforming growth factor beta 1 null mutation

in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci USA. 90:770-774.

Kusakabe, M., Cheong, P.L., Nikfar, R., McLennan, I.S. and Koishi, K. 2008. The structure of the TGF-beta latency associated peptide region determines the ability of the proprotein convertase furin to cleave TGF-betas. J Cell Biochem. 103:311-320.

Laiho, M., Saksela, O., Andreasen, P.A. and Keski-Oja, J. 1986. Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-beta. J Cell Biol. 103:2403-2410.

Lamb, T.D. 2009. Evolution of vertebrate retinal photoreception. Phil Trans R Soc B 364:2911-2924.

Lampen, A., Meyer, S. and Nau, H. 2001. Effects of receptor-selective retinoids on CYP26 gene expression and metabolism of all-trans-retinoic acid in intestinal cells. Drug Metab Dispos. 29:742-747.

Larsson, J. and Karlsson, S. 2005. The role of Smad signaling in hematopoiesis. Oncogene 24:5676-5692.

LaRue, A.C., Lansford, R. and Drake, C.J. 2003. Circulating blood island-derived cells contribute to vasculogenesis in the embryo proper. Dev Biol. 262:162-172.

LaRue, A.C., Argraves, W.S., Zile, M.H. and Drake, C.J. 2004. Critical role for retinol in the generation/differentiation of angioblasts required for embryonic blood vessel formation. Dev Dyn. 230:666-674.

Lee, K.H., Song, S.U., Hwang, T.S., Yi, Y., Oh, I.S., Lee, J.Y., Choi, K.B., Choi, M.S. and Kim, S.J. 2001. Regeneration of hyaline cartilage by cell-mediated gene therapy using transforming growth factor b1-producing fibroblasts. Hum Gene Ther. 12:1805-1813.

Lee, M.K., Pardoux, C., Hall, M.C., Lee, P.S., Warburton, D., Quing, J., Smith, S.M. and Derynck, R. 2007. TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. EMBO J. 26:3957-3967.

Letterio, J.J. and Roberts, A.B. 1996. Transforming growth factor-beta1-deficient mice: identification of isoform-specific activities in vivo. J Leukoc Biol. 59:769-774.

Levin, M., Johnson, R.L., Stern, C.D., Kuehn, M. and Tabin, C. 1995. A molecular pathway determining left–right asymmetry in chick embryogenesis. Cell 82:803-814.

Li, P., Pashmforoush, M. and Sucov, H. 2010. Retinoic acid regulates differentiation of the secondary heart field and TGFβ-mediated outflow tract septation. Dev Cell. 18:480-485.

Linask, K.K. 1992. N-cadherin localization in early heart development and polar expression of Na +,K(+)-ATPase, and integrin during pericardial coelom formation and epithelialization of the differentiating myocardium. Dev Biol. 151:213-224.

Linask, K.K. 2003. Regulation of heart morphology: current molecular and cellular perspectives on the coordinated emergence of cardiac form and function. Birth Defects Res C Embryo Today 69:14-24.

Linask, K.K. and Lash, J.W. 1988a. A role for fibronectin in the migration of avian precardiac cells. I. Dose-dependent effects of fibronectin antibody. Dev Biol. 129:315-323.

Linask, K.K. and Lash, J.W. 1988b. A role for fibronectin in the migration of avian precardiac cells. II. Rotation of the heart-forming region during different stages and its effects. Dev Biol. 129:324–329.

Lincecum, J.M., Fannon, A., Song, K., Wang, Y. and Sassoon, D.A. 1998. Msh homeobox genes regulate cadherin-mediated cell adhesion and cell-cell sorting. J Cell Biochem. 70:22-28.

Lindskog, H., Athley, E., Larsson, E., Lundin, S., Hellström, M. and Lindahl, P. 2008. New insights to vascular smooth muscle cell and pericyte differentiation of mouse embryonic stem cells in vitro. Arterioscler Thromb Vasc Biol. 26:1457-64.

Lints, T.J., Parsons, L.M., Hartley, L., Lyons, I. and Harvey, R.P. 1993. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 119:419-431.

López-Casillas, F., Cheifetz, S., Doody, J., Andres, J.L., Lane, W.S. and Massague, J. 1991. Structure and expression of the membrane protoeoglycan betaglycan, a component of the TGF-β receptor system. Cell 67:785-795.

López-Casillas, F., Payne, H.M., Andres, J.L. and Massagué, J. 1994. Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. J Cell Biol. 124:557-568.

López, S.L., Dono, R., Zeller, R. and Carrasco, A.E. 1995. Differential effects of retinoic acid and retinoid antagonist on the spatial distribution of the homeoprotein Hoxb-7 in vertebrate embryos. Dev Dyn. 204:457-471.

Luo, J., Sucov, H., Badre, J.A., Evans, R.M. and Giguere, V. 1996. Compound mutants for retinoic acid receptor (RAR) β and RARα1 reveal developmental functions for multiple RARβ isoforms. Mech Dev. 55:33-44.

Lyons, G.E. 1996. Vertebrate heart development. Curr Opin Genet Dev. 6:454-460.

MacLean, G., Li, H., Metzger, D., Chambon, P. and Petkovich, M. 2007. Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. Endocrinology 148:4560-4567.

Maden, M. 2001. Vitamin A and developing embryo. Postgrad Med J. 77:489-491.

Maden, M., Gale, E., Kostetskii, I. and Zile, M.H. 1996. Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. Curr Biol. 6:417-426.

Madisen, L., Webb, N.R., Rose, T.M., Marquardt, H., Ikeda, T., Twardzik, D., Seyedin, S. and Purchio, A.F. 1988. Transforming growth factor-beta2: cDNA cloning and sequence analysis. DNA 7:1-8.

Mahmood, R., Flanders, K.C. and Morriss-Kay, G.M. 1992. Interactions between retinoids and TGFβs in mouse morphogenesis. Development 115:67-74.

Mahmood, R, Flanders, K.C. and Morris-Kay, G.M. 1995. The effects of retinoid status on TGF beta expression during mouse embryogenesis. Anat Ambryol (Berl). 192:21-33.

Makhijani, N.S., Bischoff, D.S. and Yamaguchi, D.T. 2005. Regulation of proliferation and migration in retinoic acid treated C3H10T1/2cells by TGF-β isoforms. J Cellular Physiol. 202:304-313. NAV PILNAIS TEKSTS.

Mangelsdorf, D.J., Borgmeyer, U., Heyman, R.A., Zhou, J.Y., Ong, E.S., Oro, A.E., Kakizuka, A. and Evans, R.M. 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. Genes Dev. 6:329-344.

Mangelsdorf, D.J., Ong, E.S., Dyck, J.A. and Evans, R.M. 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature 345:224-229.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R. 1995. The nuclear receptor superfamily: the second decade. Cell 83:835-839.

Massagué, J. 1990. The transforming growth factor-beta family. Annu Rev Cell Biol. 6:597-641.

Massagué, J. 1998. TGF-beta signal transduction. Annu Rev Biochem. 67:753-791.

Massagué, J. and Chen, Y.G. 2000. Controlling TGF-beta signaling. Genes Dev. 14:627-644.

Massagué, J., Seoane, J. and Wotton, D. 2005. Smad transcription factors. Genes Dev. 19:2783-2810.

Matsuura, I., Wang, G., He, D. and Liu, F. 2005. Identification and characterization of ERK MAP kinase phosphorylation sites in Smad3. Biochemistry 44:12546-12553.

McCollum, E.V. and Davis, M. 1913. The necessity of certain lipins in the diet during growth. J Biol Chem. 15:167-175.

McCollum, E.V., Simmonds, N. and Parsons, H.T. 1921. Supplementary protein values in foods: I. The nutritive properties of animal tissues. J Biol Chem. 47:111-137.

McCormick, K.M. 2001. TGFβ2 activation status during cardiac morphogenesis. Dev Dyn. 222:17-25.

Mecha, M., Rabadán, M.A., Peña-Melián, A., Valencia, M., Mondéjar, T. and Blanco, M.J. 2008. Expression of TGF-betas in the embryonic nervous system: analysis of interbalance between isoforms. Dev Dyn. 237:1709-1717.

Mendelsohn, C., Lohnes, D., Décimo, D., Lufkin, T., LeMeur, M., Chambon, P. and Mark, M. 1994. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages if organogenesis in RAR double mutants. Development 120:2749-2771.

Mercado-Pimentel, M.E., and Runyan, R.B. 2007. Multiple transforming growth factor-beta isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. Cells Tissues Organs 185:146-156.

Mic, F., Molorkov, A., Benbrook, D.M. and Dueaster, G. 2003. Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. Proc Natl Acad Sci USA. 100:7135–7140.

Mikawa, T. and Hurtado, R. 2007. Development of the cardiac conduction system. Semin Cell Dev Biol. 18:90-100.

Miller, D.A., Lee, A., Pelton, R.W., Chen, E.Y., Moses, H.L. and Derynck, R. 1989. Murine transforming growth factor-beta 2 cDNA sequence and expression in adult tissues and embryos. Mol Endocrinol. 3:1108-1114.

Mitrani, E. and Shimoni, Y. 1989. Retinoic acid inhibits growth in agarose of early chick embryonic cells and may be involved in regulation of axis formation. Development 107:275-280.

Miyagi, M., Yokoyama, H., Shiraishi, H., Matsumoto, M. and Ishii, H. 2001. Simultaneous quantification of retinol, retinal, and retinoic acid isomers by high-performance liquid chromatography with a simple gradiation. J Chromatogr B Biomed Sci Appl. 757:365-8.

Miyazono, K., Olofsson, A., Colosetti, P. and Heldin, C.H. 1991. A role of the latent TGF-beta 1-binding protein in the assembly and secretion of TGF-beta 1. EMBO J. 10:1091-1101.

Mizoi, T., Ohtani, H., Miyazono, K., Miyazawa, M., Matsuno, S. and Nagura, H. 1993. Immunoelectron microscopic localization of transforming growth factor beta 1 and latent

transforming growth factor beta 1 binding protein in human gastrointestinal carcinomas: qualitative difference between cancer cells and stromal cells. Cancer Res. 53:183-190.

Molin, D.G.M., Poelman, R.E., DeRuiter, M.C., Azhar, M., Doetschman, T. & Gittenberger-de Groot, A.C. 2004. Transforming growth factor β -SMAD2 signaling regulates aortic arch innervation and development. Circ Res. 95:1109-1117.

Molotkova, N., Molotkov, A. and Duester, G. 2007. Role of retinoic acid during forebrain development begins late when Raldh3 generates retinoic acid in the ventral subventricular zone. Dev Biol. 303:601-610.

Montero-Balaguer, M., Swirsding, K., Orsenigo, F., Cotelli, F., Mione, M. and Dejana, E. 2009. Stable vascular connections and remodeling require full expression of VE-cadherin in zebrafish embryos. PLoS ONE 4:e5772.

Moustakas, A. and Heldin, C.-H. 2009. The regulation of TGFβ signal transduction. Development 136:3699-3714.

Mummery, C.L., Slager, H., Kruijer, W., Feijen, A., Freund, E., Koornneef, I. and van den Eijnden-van Raaij, A.J. 1990. Expression of transforming growth factor beta 2 during the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev Biol. 137 (1):161-170.

Nagao, A. 2004. Oxidative conversion of carotenoids to retinoids and other products. J Nutr. 134:237S-240S.

Nakagawa, S. and Takeichi, M. 1997. N-cadherin is crucial for heart formation in the chick embryo. Dev Growth Differ. 39:451-455.

Nakaya, Y. and Sheng, G. 2009. An amicable separation: Chick's way of doing EMT. Cell Adh Migr. 3:160-163.

Napoli, J. and Race, K. 1988. Biogenesis of retinoic acid from b-carotene. J Biol Chem. 263:17372-17377.

Napoli, J., Pramanik, B.C., Williams, J.B., Dawson, M.I. and Hobbs, P.D. 1985. Quantification of retinoic acid by gas-liquid chromatography - mass spectrometry: total versus all-trans-retinoic acid in human plasma. J Lipid Res. 26:387-392.

Napoli, J.L. 1993. Biosynthesis and metabolism of retinoic acid: roles of CRBP and CRABP in retinoic acid: roles of CRBP and CRABP in retinoic acid homeostasis. J Nutr. 123:362-366.

Napoli, J.L. 1996. Retinoic acid biosynthesis and metabolism. FASEB J. 10:993-1001.

Naveena, B.J., Indranie, C, Altaf, M., Vernon, E.S. and Chinthalapally, V.R. 2008. β-ionone inhibits colonic aberrant crypt foci formation in rats, suppresses cell growth, and induces retinoid X receptor-α in human colon cancer. Mol Cancer Ther. 7:181-190.

Nemer, M. 2008. Genetic insigths into normal and abnormal heart development. Cardiovascular Pathology 17:48-54.

New, D.A.T. 1955. A new technique for the cultivation of the chick embryo in vitro. J Embryol Expl Morph. 3:326-331.

Niederreither, K. and Dolle, P. 2008. Retinoic acid in development: towards an integrated view. Nat Rev Genetics 9:541-553.

Niederreither, K., Vermont, J., Messaddeg, N., Schuhbaur, B., Chambon, P. and Dolle, P. 2001. Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. Development 128:1019-1031.

Noguchi, A., Nawa, M., Aiso, S., Okamoto, K. and Matsuoka, M. 2010. Transforming growth factor beta2 level is elevated in neurons of Alzheimer's disease brains. Int J Neurosci. 120:168-175.

Noma, T., Glick, A.B., Geiser, A.G., O'Reilly, M.A., Miller, J., Roberts, A.B. and Sporn, M.B. 1991. Molecular cloning and structure of the human transforming growth factor-β2 gene promoter. Growth Factors 4,:247-255.

Nugent, P. and Greene, R.M. 1994. Interactions between the transforming growth factor beta (TGF beta) and retinoic acid signal transduction pathways in murine embryonic palatal cells. Differentiation 58:149-155.

Nugent, P., Ma, L. and Greene, R.M. 1998. Differential expression and biological activity of retinoic acid-induced TGFbeta isoforms in embryonic palate mesenchymal cells. J Cell Physiol 177:36-46.

Nugent, P., Potchinsky, M., Lafferty, C. and Greene, R.M. 1995. TGF-beta modulates the expression of retinoic acid-induced RAR-beta in primary cultures of embryonic palate cells. Exp Cell Res 220:495-500.

Nunes, I., Gleizes, P.E., Metz, C.N. and Rifkin, D.B. 1997. Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. J Cell Biol. 136:1151-1163.

Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H. and Reichmann, E. 1998. TGFb is necessary for carcinoma cell invasiveness and metastasis. Curr Biol. 8:1243-1252.

Oka, M., Iwata, C., Suzuki, H.I., Kiyono, K., Morishita, Y., Watabe, T., Komuro, A., Kano, M.R. and Miyazono, K. 2008. Inhibition of endogenous TGF-β signaling enhances lymphangiogenesis. Blood 111:4571 - 4579.

Olofsson, A., Miyazono, K., Kanzaki, T., Colosetti, P., Engström, U. and Heldin, C.H. 1992. Transforming growth factor-beta 1, -beta 2, and -beta 3 secreted by a human glioblastoma cell line. Identification of small and different forms of large latent complexes. J Biol Chem. 267:19482-19488.

Olson, J.A. 1999. Carotenoids and human health. Arch Latinoam Nutr. 49:7S-11S.

Onai, T., Lin, H.C., Shubert, M., Koo, D., Osborne, P.W., Alvarez, S., Alvarez, R., Holland, N.D. and Holland, L.Z. 2009. Retinoic acid and Wnt/beta-catenin have coplmentary roles in anterior/posterior patterning embryos of the basal chordate amphioxus. Dev Biol. 332(2): 223-233.

Osmond, M.K., Butler, A.J., Voon, F.C.T. and Bellairs, R. 1991. The effects of retinoic acid on heart formation in the early chick embryo. Development 113:1405-1417.

Ott, D.B. and Lachance, P.A. 1979. Retinoic acid - a review. Am J Clin Nutr. 32:2522-2531.

Ouyang, G., Wang, Z., Fang, X., Liu, J. and Yang, C.J. 2010. Molecular signaling of the epithelial to mesenchymal transition in generating and maintaining cancer stem cells. Cell Mol Life Sci. 67:2605-18.

Padgett, R.W., Savage, C. and Das, P. 1997. Genetic and biochemical analysis of TGF beta signal transduction. Cytokine Growth Factor Rev. 8:1-9.

Parés, X., Farrés, J., Kedishvili, N. and Duester, G. 2008. Medium-chain and short-chain dehydrogenases/reductases in retinoid metabolism. Cell Mol Life Sci. 65:3936-3949.

Parker, P. 1996. Absorption, metabolism and transport of carotenoids. FASEB J. 10:542-551.

Patan, S. 2000. Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. Journal of Neuro-Oncology 50:1-15.

Pepper, M. 1999.Transforming growth factor-beta:vasculogenesis, angiogenesis, and vessel wall integrity. Cytokine and Growth Factor Rev. 8:21-43.

Perlmann, T. 2002. Retinoid metabolism: a balancing act. Nat Gen. 31:7-8.

Petkovich, M., Brand, N.J., Krust, A. and Chambon, P. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330:444-450.

Petrie, K., Zelent, A. and Waxman, S. 2009. Differentiation therapy of acute myeloid leukemia: past, present and future. Curr Opin Hematol. 16:84-91.

Pfahl, M. 1993. Signal transduction by retinoid receptors. Skin Pharmacol. 6 Supppl 1:8-16.

Piek, E., Moustakas, A., Kurisaki, A., Heldin, C.H., Ireland, H., Akurs, R.J. and Balmain, A. 1999. TGF-b type I receptor/ALK5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NmuMG breast epithelial cells. J Cell Sci. 112:4557–4568.

Poole, T.J., Finkelstein, E.B. and Cox, C.M. 2001. The role of FGF and VEGF in angioblast induction and migration during vascular development. Dev Dyn. 220:1-17.

Proetzel, G., Pawlowski, S.A., Wiles, M.V., Yin, M., Boivin, G.P., Howles, P.N., Ding, J., Ferguson, M.W. and Doetschman, T. 1995. Transforming growth factor-beta 3 is required for secondary palate fusion. Nat Genet. 11:409-414.

Redondo, C., Vouropoulou, M., Evans, J. and Findlay, J.B. 2008. Identification of the retinol binding protein (RBP) interaction site and function state of RBPs for the membrane receptor. FASEB J. 22:1043-1054.

Reijntjes, S., Blentic, A., Gale, E. and Maden, M. 2005. The control of morphogen signaling: regulation of the synthesis and catabolism of retinoic acid in the developing embryo. Dev Biol. 285:224-237.

Ribeiro, S.M., Poczatek, M., Schultz-Cherry, S., Villain, M. and Murphy-Ullrich, J.E. 1999. The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent transforming growth factor-b. J Biol Chem. 274:13586-13593.

Ribes, V., Wang, Z., Dolle, P. and Niederreither, K. 2005. Retinaldehyde dehydrogenase 2 (RALDH2)-mediated retinoic acid synthesis regulates early mouse embryonic forebrain development by controlling FGF and sonic hedgehog signaling. Development 133:351-361.

Riekstina, U., Cakstina, I., Parfejevs, V., Hoogduijn, M., Jankovskis, G., Muiznieks, I., Muceniece, R. and Ancans, J. 2009. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. Stem Cell Rev. 5(4):378-386.

Rifkin, D.B., 2004. Latent transforming growth factor-β (TGF-β) binding proteins: orchestrators of TGF-β availability. J Biol Chem. 280:7409-7412.

Roberts, A.B. and Sport, M.B. 1992. Mechanistic interrelationships between two superfamilies: the streoid/retinoid receptors and transforming growth factor-beta. Cancer Rev. 14:205-220.

Rochette-Egly, C. and Germain, P. 2009. Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs). Nuclear Receptor Signaling 7:e005.

Roesler, W.J., Vandenbark, G.R. and Hanson, R.W. 1988. Cyclic AMP and the induction of eukaryotic gene transcription. J Biol Chem. 263:9063-9066.

Roman, B.L. and Weinstein, B.M. 2000. Building the vertebrate vasculature: research is going swimmingly. BioEssays 22:882-893.

Romano, L.A. and Runyan, R.B. 2000. Slug is an essential target of TGFβ2 signaling in the developing chicken heart. Dev Biol. 223:91-102.

Romeih, M., Cakstina, I. and Zile, M.H. 2009. Retinoic acid is a negative physiological regulator of N-cadherin during early avian heart morphogenesis. Dev Growth Differ. 51: 753-767.

Romeih, M., Cui, J., Michaille, J.-J., Jiang, W. and Zile, M.H. 2003. Function of RARγ and RARα2 at the initiation of retinoid signaling is essential for avian embryo survival and for distinct events in cardiac morphogenesis. Dev Dyn. 228:697–708.

Roos, T.C., Jugert, F.K., Merk, H.F. and Bickers, D.R. 1998. Retinoid metabolism in the skin. Pharmacol rev. 50:315-333.

Ross, S.A., McCaffery, P.J., Drager, U.C. and De Luca, L.M. 2000. Retinoids in embryonal development. Physiol Rev. 80:1021-1054.

Rossant, J. and Howard, L. 2002. Signaling pathways in vascular development. Annu Rev Cell Dev Biol. 18:541-573.

Rotzer, D., Roth, M., Lutz, M., Lindemann, D., Sebald, W. and Knaus, P. 2001. Type III TGF-beta receptor-independent signalling of TGF-beta2 via TbetaRII-B, an alternatively spliced TGF-beta type II receptor. EMBO J. 20:480-490.

Rozario, T. and DeSimone, D.W. 2010. The extracellular matrix in development and morphogenesis: A dynamic view. Dev Biol. 341:126-40.

Runyan, R.B. and Markwald, R.R. 1983. Invasion of mesenchyme into three-dimensional collagen gels: a regional and temporal analysis of interaction in embryonic heart tissue. Dev Biol. 95:108–114.

Saharinen, J., Taipale, J., Monni, O. and Keski-Oja, J. 1998. Identification and characterization of a new latent transforming growth factor-beta-binding protein, LTBP-4. J Biol Chem. 273:18459-18469.

Saika, S. 2006. TGFbeta pathobiology in the eye. Lab Invest. 86:106-115.

Salbert, G., Fanjul, A., Piedrafita, F.J., Lu, X.P., Kin, S.J., Tran, P. and Pfahl, M. 1993. Retinoic acid receptors ans retinoid X receptor-alpha down-regulates the transforming growth factor-beta 1 promoter by antagonizing AP-1 activity. Mol Endocrinol. 7:1347-1356.

Sanford, L.P., Ormsby, I., Gittenberger-de Groot, A.C., Sariola, H., Friedman, R., Boivin, G.P., Cardell, E.L. and Doetschman, T. 1997. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development 124:2659-2670.

Satokata, I. and Maas, R. 1994. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet. 6:348-356.

Schlange, T., Andrée, B., Arnold, H.H. and Brand, T. 2000. BMP2 is required for early heart development during a distinct time period. Mech Dev. 91:259-270.

Schlueter, J. and Brand, T. 2009. A right-sided pathway involving FGF8/Snai1 controls asymmetric development of the proepicardium in the chick embryo. Proc Natl Acad Sci USA. 106:7485-7490.

Schlunegger, M.P. and Grütter, M.G. 1992. An unusual feature revealed by the crystal structure at 2.2 A resolution of human transforming growth factor-beta 2. Nature 358:430-434.

Schule, R., Rangarjan, P., Yang, N., Kliewer, S., Ransone, L., Bolado, J., Verma, I.M. and Evans R.M. 1991. Retinoic acid is a negative regulator of AP-1-responsive genes. Proc Natl Acad Sci USA. 88:6092-6096.

Scita, G., Darwiche, N., Greenwald, E., Rosenberg, M., Politi, K. and De Luca, L.M. 1996. Retinoic acid down-regulation of fibronectin and retinoic acid receptor alpha proteins in NIH-3T3 cells. Blocks of this response by ras transformation. J Biol Chem. 271:6502-8.

Sekelsky, J.J., Newfeld, S.J., Raftery, L.A., Chartoff, E.H. and Gelbart, W.M. 1995. Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. Genetics 139:1347-1358.

Selleck, M.A.J. 1996. Culture and microsurgical manipulation of the early avian embryo. In: Bronner-Fraser, M., editor. Methods in Cell Biology. vol.51. Methods in Avian Embryology. pp. 1-21. New York: Academic Press.

Seton-Rogers, S.E., Lu, Y., Hines, L.M., Koundinya, M., LaBaer, J., Muthuswamy, S.K. and Brugge, J.S. 2004. Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. Proc Natl Acad Sci USA. 101:1257-1262.

Shalaby, F., Ho, J., Stanford, W.L., Fischer, K.D., Schuh, A.C., Schwartz, L., Bernstein, A. and Rossant, J. 1997. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell 89:981-990.

Shaulian, E. and Karin, M. 2002. AP-1 as a regulator of cell life and death. Nat Cell Biol. 4:E131-E136.

Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annuziata, N. and Doetschman, T. 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature 359:693-699.

Sidell, N., Feng, Y., Hao, L., Wu, J., Yu, J., Kane, M.A., Napoli, J.L. and Taylor, R.N. 2010. Retinoic acid is a cofactor for translational regulation of vascular endothelial growth factor in human endometrial stromal cells. Mol Endocrinol. 24:148-160.

Singh, B., Murphy, R.F., Ding, X.Z., Roginsky, A.B., Bell, R.H.Jr. and Adrian, T.E. 2007. On the role of transforming growth factor-beta in the growth inhibitory effects of retinoic acid in human pancreatic cancer cells. Mol Cancer. 6:82.

Sirard, C., Kim, S., Mirtsos, C., Tadich, P., Hoodless, P.A., Itié, A., Maxon, R., Wrana, J.L. and Mak, T.W. 2000. Targeted disruption in murine cells reveals variable requirement for Smad4 in transforming growth factor beta-related signaling. J Biol Chem. 275:2063-70.

Skromne, I. and Stern, C.D. 2001. Interactions between Wnt and Vg1 signaling pathways initiate primitive streak formation in the chick embryo. Development 128:2915-2927.

Sorrentino, A., Thakur, N., Grimsby, S., Marcusson, A., von Bulow., V., Schuster, N., Zhang, S., Heldin, C.H. and Landström, M. 2008. The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat Cell Biol. 10:1199-1207.

Sporn, M.B. and Roberts, A.B. 1992. Transforming growth factor-beta: recent progress and new challenges. J Cell Biol. 119:1017-1021.

St.Amand, T., Ra, J., Zhang, Y., Hu, Y., Baber, S. I., Qui, M.-S. and Chen, Y.-P. 1998. Cloning and expression pattern of chicken Pitx2: A new component in the SHH signaling pathway controlling embryonic heart looping. Biochem Biophys Res Commun. 247:100-105.

Stehlin-Gaon, C., Willmann, D., Zeyer, D., Sanglier, S., Van Dorsselaer, A., Renaud, J.P. Moras, D. and Schule R. 2003. All-trans retinoic acid is ligand for the orphan nuclear receptor ROR beta. Nat Struct Biol. 10:820-825.

Stuckmann, I., Evans, S. and Lassar, A.B. 2003. Erythropoietin and retinoic acid, secreted from the epicardium, are required for cardiac myocyte proliferation. Dev Biol 255:334-349.

Sucov, H.M., Dyson, E., Gumeringer, C.L., Price, J., Chien, K.R. and Evans, R.M. 1994. RXRα mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. Genes Dev. 8:1007-1018.

Sun, S.Y. and Lotan, R. 2002. Retinoids and their receptors in cancer development and chemoprevention. Crit Rev Oncol Hematol. 41:41-55.

Taipale, J., Lohi, J., Saarinen, J., Kovanen, P.T. and Keski-Oja, J. 1995. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-b1 from the extracellular matrix of cultured human epithelial and endothelial cells. J Biol Chem. 270:4689-4696.

Taipale, J., Miyazini, K., Heldin, C.H. and Keski-Oja, J. 1994. Latent transforming growth factor- beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. J Cell Biol. 124:171-181.

Taipale, J., Saharinen, J. and Keski-Oja, J. 1998. Extracellular matrix-associated transforming growth factor-beta: role in cancer cell growth and invasion. Adv Cancer Res. 75:87-134.

Tavian, M. and Peault, B. 2005. Embryonic development of the human hematopoietic system. Int J Dev Biol. 49:243-250.

Theil, T., Aydin, S., Koch, S., Grotewold, L. and Ruther, U. 2002. Wnt and Bmp signaling cooperatively regulate graded Emx2 expression in the dorsal telencephalon. Development 129:3045-3054.

Thomas, P.S., Kasahara, H., Edmonson, A.M., Izumo, S., Yacoub, M.H., Barton, P.J. and Gourdie, R.G. 2001. Elevated expression of Nkx-2.5 in developing myocardial conduction cells. Anat Rec. 263:307-313.

Trinh, L.A. and Stainier, D.Y. 2004. Fibronectin regulates epithelial organization during myocardial migration in zebrafish. Dev Cell 6:371-382.

Twal, W., Roze, L. and Zile, M.H. 1995. Anti-retinoic acid monoclonal antibody localizes all-trans-retinoic acid in target cells and blocks normal development in early quail embryo. Dev Biol. 168:225-234.

Tyssandier, V., Reboul, E., Dumas, J.F., Bouteloup-Demange, C., Armands, M., Marcands, J., Sallas, M. and Borel, P. 2003. Processing of vegetable-borne carotenoids in the human stomach and duodenum. Am J Physiol Gastrointest Liver Physiol. 284:G913-G923.

Ulven, S.M., Gundersen, T.E., Weedon, M.S., Landaas, V.O., Sakhi, A.K., Fromm, S.H., Geronimo, B.A., Moskaug, J.O. and Blomhoff, R. 2000. Identification of endogenous retinoids, enzymes, binding proteins, and receptors during early postimplantation development in mouse: important role of retinal dehydrogenase type 2 in synthesis of all-trans-retinoic acid. Dev Biol. 220:379-391.

Ventura, J.J., Kennedy, N.J., Flavell, R.A. and Davis, R.J. JNK regulates autocrine expression of TGF-beta1. Mol Cell 15:269-278.

Verrecchia, F., Chu, M.L. and Mauviel, A. 2001. Identification of novel TGF-beta /Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. J Biol Chem. 276:17058-17062.

Vincent, E.B., Runyan, R.B. and Weeks, D.L. 1998. Production of the transforming growth factor-β binding protein endoglin is regulated during chick heart development. Dev Dyn. 213:237-247.

Virågh, S., Szabó, E. and Challice, C.E. 1989. Formation of the primitive myo- and endocardial tubes in the chicken embryo. J Mol Cell Cardiol. 21:123-137.

Wada, T., Kang, H.S., Jetten, A.M. and Xie, W. 2008. The emerging role of nuclear receptor RORα and its crosstalk with LXR in xeno- and endobiotic gene regulation. Exp Biol Med. 233:1191-1201.

Wagner, M. and Siddiqui, M.A.Q. 2007.a Signal transduction in early heart development (I): cardiogenic induction and heart tube formation. Exp Biol Med. 232:852-865.

Wagner, M. and Siddiqui, M.A.Q. 2007.b Signal transduction in early heart development (II): ventricular chamber specification, trabeculation, and heart valve formation. Exp Biol Med. 232:866-880.

Walls, J.R., Coultas, L., Rossant, J. and Henkelman, R.M. 2008. Three-dimensional analysis of vascular development in the mouse embryo. PLoS ONE 3:e2853.

Wang, X.F., Lin, H.Y., Ng-Eaton, E., Downward, J., Lodish, H.F. and Weinberg, R.A. 1991. Expression cloning and characterization of the TGF-beta type III receptor. Cell 67:797-805.

Watt, F.M. and Hodivala, K.J. 1994. Cell adhesion. Fibronectin and integrin knockouts come unstuck. Curr Biol. 4:270-272.

Webb, N.R., Madisen, L., Rose, T.M. and Purchio, A.F. 1988. Structural and sequence analysis of TGF-beta 2 cDNA clones predicts two different precursor proteins produced by alternative mRNA splicing. DNA 7:493-497.

Weinstein, B. 1999. What guides embryonic blood vessel formation? Dev Dyn.215:2-11.

Weinstein, M., Yang, X. and Deng, C. 2000. Functions of mammalian Smad genes as revealed by targeted gene disruption in mice. Cytokine Growth Factor Rev. 11:49-58.

White, J.A., Beckett-Jones, B., Guo, Y.D., Dilworth, J., Bonasoro, J., Jones, G. and Petkovich, M. 1997. cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450 (CYP26). J Biol Cehm. 272:18538-18541.

Whitman, M. and Raftery, L. 2005. TGFβ signaling at the summit. Development 132:4205-4210.

Willert, J., Epping, M., Pollack, J.R., Brown, P.O. and Nusse, R. 2002. A transcriptional response to Wnt protein in human embryonic carcinoma cells. BMC Dev Biol. 2:8.

Wilson, J.G., Roth, C.B. and Warkany, J. 1953. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various time during gestation. Am J Anat. 92:189-217.

Winer, J., Jung, C.K., Shackel, I. and Williams, P.M. 1999. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem. 270:41-49.

Wood, H., Pall, G. and Morriss-Kay, G. 1994. Exposure to retinoic acid before or after the onset of somitogenesis reveals separate effects on rhombomeric segmentation and 3' HoxB gene expression domains. Development 120:2279-2285.

Wrana, J.L., Attisano, L., Weiser, R., Ventura, F. and Massagué, J. 1994. Mechanism of activation of the TGF-beta receptor. Nature 370:341-347.

Wu, M.Y. and Hill, C.S. 2009. TGF-β superfamily signaling in embryonic development and homeostasis. Developmental Cell 16:329-343.

Xiao, J.H., Durand, B., Chambon, P. and Voorhees, J.J. 1995. Endogenous retinoic acid receptor (RAR)-retinoid X receptor (RXR) heterodimers are the major functional forms regulating retinoid-responsive elements in adult human keratinocytes. J Biol Chem. 270:3001-3011.

Xu, L., Lamouille, S. and Derynck, R. 2009. TGFβ-induced epithelial to mesenchymal transition. Cell Res. 19:156-172.

Yang, J. and Weinberg, R. 2008. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell 14:818-829.

Yoshizawa, M., Miyazaki, H. and Kojima, S. 1998. Retinoids potentiate transforming growth factor-β activity in bovine endothelial cells through up-regulating the expression of transforming growth factor-beta receptors. J Cellular Physiol. 176:565-573.

Yu, X., He, F., Zhang, T., Espinoza-Lewis, R.A., Lin, L., Yang, J. and Chen, Y. 2008. Cerberus functions as a BMP agonist to synergistically induce Nodal expression during left-right axis determination in the chick embryo. Dev Dyn. 237:3613-3623.

Zhang, H. and Bradley, A. 1996. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122:2977-2986.

Zhang, H., Toyofuku, T., Kamei, J. and Hori, M. 2003. GATA-4 regulates cardiac morphogenesis through transactivation of the N-cadherin gene. Biochem Biophys Res Commun. 312: 1033-1038.

Zhou, H.R., Abouzied, M.M. and Zile, M.H. 1991. Production of a hybridoma cell line secreting retinoic acid- specific monoclonal antibody. J Immunol Methods 138:211-223.

Zhou, X.F., Shen, X.Q. and Shemshedini, L. 1999. Ligand-activated retinoic acid receptor inhibits AP-1 transactivation by disrupting c-Jun/c-Fos dimerization. Mol Endocrinol. 13:276-285..

Zile, M.H. 2004. Vitamin A requirement for early cardiovascular morphogenesis specification in the vertebrate embryo: insights from the avian embryo. J Exptl Biol Med. 229:598-606.

Zile, M.H. 2010. Vitamin A-not for your eyes only: requirement for heart formation begins early in embryogenesis. Nutrients 2:532-550.

Zile, M.H., Kostetskii, I., Yuan, S., Kostetskaia, E., St. Amand, T.R., Chen, Y. and Jiang, W. 2000. Retinoid signaling is required to complete the vertebrate cardiac left/right asymmetry pathway. Dev Biol. 223:323-338.

Ziouzenkova, O. and Plutzky, J. 2008. Retinoid metabolism and nuclear receptor responses: new insights into coordinated regulation of the PPAR-RXR complex. FEBS Letters 582:32-38.

APPENDIX

Appendix 1

The PCR and real-time PCR oligonucleotide sequences

Name	Sequence (5'- 3')	Product size (bp)	Gene Bank ref.nr.
Chick N-cadherin F	agccgacaacgaccctacag	70	NM_001001615.1
Chick N-cadherin R	tggagccgcttccttcatag		
Chick TGFβ2 F	caataggaaaaaatacagtgggaagac	84	NM_001031045.2
Chick TGFβ2 R	actgggctgttgcgactca		
chick-b-actin F	ctgaaccccaaagccaacag	214	X00182.1
chick-b-actin R	ccagatccagacggaggatg		
chick TGFb2 F	cagtgggaagaccccacatc	185	X59080.1
chick TGFb2 R	tgaatccatttccagccaag		
chick FN1 F	cgcccctaccactctgacac	175	XM_421868.2
chick FN1 R	cagctctgcaacgtcctcct		
chick NEO F	tccggatagctgccatgact	161	U07644.1
chick NEO R	tggagtccagctcaccacaa		
chick HoxB1 F	aacctctcgccttccctaaa	191	NM_001080859
chick HoxB1 R	agctgcttggtggtgaagtt		
chick VEGF F	gcagagcgcggagttgtc	127	NM_001110355
chick VEGF R	gtccaccagggtctcaattgtc		
h b-actin F	agtgtgacgtggacatccg	207	NM_001101
h b-actin R	aatctcatcttgttttctgcgc		
h GAPDH F	gtcatccctgagctagacgg	171	NM_002046
h GAPDH R	gggtcttactccttggaggc		
h PGK1 F	cttaaggtgctcaacaacatgg	119	NM_000291
h PGK1 R	acaggcaaggtaatcttcacac		
h TGFb2 F	ccggaggtgatttccatcta	221	NM_003238
h TGFb2 R	ctccattgctgagacgtcaa		
h FN1 F	ggagttgattataccatcactg	259	NM_002026
h FN1 R	tttctgtttgatctggacct		
h NEO F	gggacccatgtctgaagctg	149	NM_002499
h NEO R	tactgccgctcattggaggt		
h c-Myc F	tgacctgaaagaccgaccat	181	NM_001018139
h c-Myc R	gccaacctgaatgcagaagt		
h b-cat F	gcagaaaatggttgccttgc	135	NM_001098210
h b-cat R	aaagcttggggtccaccact		
h CD44 F	ggctgatcatcttggcatcc	172	NM_001001391
h CD44 R	tgagacttgctggcctctcc		
h lam B1 F	tccaccacagaacccaacag	193	NM_002291
h lam B1 R	ggggtgttccacaggtcatt		

Appendix 2

PCR oligonucleotide sequences used for characterization of human MSCs

Name	Sequence (5’ - 3’)	Product size (bp)	GeneBank reference nr.
oct4 F	gtggaggaagctgacaacaa	119	NM_002701.3
oct4 R	attctccaggttgcctctca		
nanog F	cctgtgatttgtgggcctg	77	NM_024865.2
nanog R	gacagtctccgtgtgaggcat		
sox2 F	gtatcaggagttgtcaaggcagag	77	NM_003106.2
sox2 R	tcctagtcttaaagaggcagcaaac		
nestin F	gccctgaccactccagttta	220	NM_006617.1
nestin R	ggagtcctggatttccttcc		
GATA4 F	tcatctcactacgggcacag	233	NM_002052.2
GATA4 R	gggaagagggaagattacgc		
TGF-β1 F	gcgtgctaatggtggaaac	275	NM_000660.3
TGF-β1 R	cggtgacatcaaaagataaccac		
CD90 F	ctagtggaccagagccttcg	333	NM_006288.2
CD90 R	tggagtgcacacgtgtaggt		
CD105 F	tgccactggacacaggataa	204	NM_000118.1
CD105 R	ccttcgagacctggctagtg		
β-actin F	tccttcctgggcatggag	207	NM_001101.2
β-actin R	aggaggagcaatgatcttgatctt		