Developmental Origin and Molecular Regulation of Vascular Smooth Muscle Cells

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University of Gothenburg 2008

ISBN 978-91-628-7637-1

Printed by Intellecta DocuSys AB
Västra Frölunda, Sweden, 2008

Abstract

Several pathologies of the vascular system have been suggested to be dependent on the smooth muscle cells (SMCs) that build up the vessel wall.

Aortic SMCs have been proposed to derive from lateral plate mesoderm. It has further been suggested that induction of SMC differentiation is confined to the ventral side of the aorta and that cells later migrate to the dorsal side. In this thesis, the developmental origin of aortic SMCs was investigated using recombination-based lineage tracing in mice. It was shown that aortic SMCs are derived from the somites and not from lateral plate mesoderm. Moreover, vascular SMCs are not recruited by a ventral-to-dorsal migration. Lateral plate mesoderm-derived SMCs on the ventral side of the aorta were shown to express SMC markers early in development. It was however demonstrated that these cells are replaced by SMCs of somitic origin at E10.5.

Lipoma preferred partner (LPP) has recently been identified as a SMC marker involved in cell migration. In this thesis, the transcriptional regulation of the LPP gene was studied. In particular it was investigated whether LPP transcription is dependent on serum response factor (SRF)/myocardin. With bioinformatic tools, an alternative transcriptional promoter was predicted within the LPP gene. This promoter was further analyzed using quantitative RT-PCR, chromatin immunoprecipitation, electrophoretic mobility-shift assays, luciferase reporter experiments and SRF-deficient cells/tissues. It was demonstrated that the alternative promoter binds SRF in vitro. It was also shown that it has transcriptional capacity, which is dependent on SRF/myocardin. The alternative promoter directs LPP expression in SMCs in vivo.

Finally, a carotid artery ligation model was used in this thesis to investigate the proposed roles of angiotensin II (Ang II) and platelet-derived growth factor B (PDGF-B) in neointimal hyperplasia. Experiments were performed in wild type mice and PDGF-B retention motif knockout mice. It was shown that PDGF-B mRNA was increased by carotid artery ligation while expression of PDGF receptor β was unaffected. The ligation induced a neointima formation that was further accelerated by Ang II administration. Neointima formation was unaffected by knockout of the PDGF-B retention motif or inhibition of the PDGF receptor β.

Key words: smooth muscle cell, aorta, cell origin, lateral plate mesoderm, paraxial mesoderm, lipoma preferred partner, serum response factor, neointimal hyperplasia, angiotensin II, platelet-derived growth factor-B.

List of publications

This thesis is based on the following papers, in the text referred to by their roman numerals:

I. Wasteson P, Johansson BR, Jukkola T, Breuer S, Akyürek LM, Partanen J, Lindahl P: **Developmental origin of smooth muscle cells in the descending aorta in mice.** *Development (2008) May;135(10):1823-32*

II. Petit MM, Lindskog H, Larsson E, Wasteson P, Athley E, Breuer S, Angstenberger M, Hertfelder D, Mattsson E, Nordheim A, Nelander S, Lindahl P: **Smooth muscle expression of lipoma preferred partner is mediated by an alternative intronic promoter that is regulated by serum response factor/myocardin.** *Circ Res. (2008) Jul 3;103(1):61-9*

III. Nyström HC, Johansson ME, Wasteson P, Lindblom P, Betsholtz C, Gan L, Lindahl P, Bergström G: **Neointimal hyperplasia of the mouse carotid artery – role of Ang II and PDGF-B.** *Manuscript*

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List of abbreviations

ACLP	aortic carboxypeptidase-like protein
Ang II	angiotensin II
C-α-MHC	cardiac-α-myosin heavy chain
CArG	$CC(A/T)_6GG$
CD31	cluster of differentiation 31, PECAM
ChIP	chromatin immunoprecipitation
Cre	causes recombination
CRP	cystein-rich protein
E	embryonic day
Edg-1	endothelial differentiation gene-1
Elk-1	member of ETS oncogene family
EMSA	electrophoretic mobility shift assay
ES	embryonic stem
FHL	four-and-a-half LIM
flk1	VEGF receptor 2
flox	flanked by lox
HRC	histidine-rich calcium-binding protein
LDL	low-density lipoprotein
LIM	LIN-11 Isl1 MEC-3
LIMD1	LIM-domains-containing protein 1
loxP	locus of crossing over
LPP	lipoma preferred partner
MADS	MCM-1 Agamous Deficiens SRF
MEF	mouse embryonic fibroblast
MRTF	myocardin-related transcription family
NES	nuclear export signal
P	postnatal day
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR-β	PDGF receptor β

PEA3	polyomavirus enhancer activator 3
PECAM	platelet/endothelial cell adhesion molecule, CD31
RetKO	retention motif knockout
ROSA	reverse orientation splice acceptor
ROSA26	ROSA 26 reporter strain
R26R	ROSA 26 reporter
RT-PCR	reverse transcriptase-polymerase chain reaction
SAP	SAF-A/B Acinus PIAS
siRNA	small interfering RNA
SM α-actin	smooth muscle α-actin
SMC	smooth muscle cell
SM-MHC	smooth muscle myosin heavy chain
SM22α	smooth muscle 22 kDa protein α-isoform
SRE	serum response element
SRF	serum response factor
TEM	transmission electron microscopy
TGF-β	transforming growth factor-β
Tie1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TRIP6	thyroid receptor interacting protein 6
TSS	transcription start site
VEGF	vascular endothelial growth factor
VENT	ventrally emigrating neural tube
Wnt1	wingless-type MMTV integration site family, member 1
WT1	Wilms' tumour suppressor gene
WTIP	WT1 interacting protein

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1. General aims

Diseases involving the cardiovascular system are common. To reveal the background of complex pathological conditions like atherosclerosis or tumour angiogenesis, it is necessary to understand normal circulatory physiology and normal vessel development. The smooth muscle cell (SMC) has appeared as one of the most interesting and perhaps most studied cells in this context. SMCs are defined on morphological criteria and on the ability to contract independently of voluntary control. SMCs can be more or less mature and display various states of differentiation. The histological SMC definition should therefore be supplemented by a characterization based on the presence of certain SMC markers. Today, several different SMC markers are known. Previously in our group, lipoma preferred partner (LPP) was described as SMC-specific. In this thesis, the transcriptional regulation of the LPP gene was studied. In particular it was investigated whether LPP expression is regulated by serum response factor (SRF). However, validation of smooth muscle-specific marker genes and descriptions of their molecular regulation will be fruitful only if morphogenesis is known in detail. In this thesis, we therefore investigated the developmental origin of SMCs in the descending aorta, in order to map some of the morphological events that take place around SMC induction. Finally in this thesis, we used a mouse carotid artery ligation model to investigate the roles of platelet-derived growth factor B (PDGF-B) and angiotensin II (Ang II) in an atherosclerosis-like situation.

2. Background

2.1. Blood vessels

2.1.1. Blood vessel morphology

2.1.1.1. Introduction

Since diffusion between cells only can appear within a radius of less than 1 mm, animals with larger bodies require a circulatory system to survive. Blood vessels can be found throughout the body and are to serve two major functions. Firstly, they provide the tissues with nutrients and oxygen and remove waste products. Secondly, they act as medium and transport routes for signaling macromolecules and hormones.

2.1.1.2. Vessel wall composition

Besides matrix components, mature blood vessels consist principally of endothelial cells and mural cells. Mural cells are contractile support cells that can be either solitary, smooth muscle-like pericytes in the finest diameter blood vessels, or vascular SMCs - organized in concentric layers in arteries and veins. Vessels that lack adequate amount of supportive mural cells will be dysfunctional because of endothelial changes, capillary dilation (microaneurysms) and rupture (Carmeliet, Mackman et al. 1996), (Lindahl, Johansson et al. 1997).

2.1.1.3. Vessel wall organization

Veins and arteries are organized in three layers: the intima, the media and the adventitia. The inner lining consists of endothelial cells. The so called tunica intima is the combination of endothelium and connective tissue on the luminal side of the internal elastic lamina. In a normal artery, the tunica intima contains a matrix of collagen, proteoglycans and small amounts of elastin. Between the endothelium and the internal elastic lamina are occasional SMCs in a normal artery (Ross and Glomset 1973). Inflammation, as part of atherosclerosis or restenosis following angioplasty,

stimulates proliferation and migration of SMCs in this area. *Neointima* is the pathological, thickened layer of arterial intima formed by migration and proliferation of cells after blood vessel grafting, angioplasty or in atherosclerosis. The internal elastic lamina is composed of an elastin layer with gaps (fenestrae) that allows substances to diffuse through. Under the internal elastic lamina lies the media. It consists of concentric layers of vascular SMCs, responsible for the tonus of the vessel wall. Adjacent to the media is the adventitia, composed essentially of collagen I and elastic fibers. Larger arteries also have adventitial fibroblasts. In larger vessels an external elastic lamina can furthermore be found between the adventitia and the media (Junqueira 1995).

2.1.2. SMC properties

2.1.2.1. SMCs exert contraction and synthesize extracellular matrix

SMCs are contractile cells found in different organ systems like the vasculature, the urogenital system, the airways and the digestive tract. SMCs have two major tasks in the body: they exert contractile abilities and synthesize structural components of the extracellular matrix. Such components include elastic fibers, collagens, adhesive glycoproteins, proteoglycans and hyaluronan. Compared with other muscles, SMCs have special smooth muscle forms of actin and myosin that build up the myofilaments. These myofilaments are anchored to cell-matrix adhesions and to so called dense bodies in the cytoplasm.

2.1.2.2. SMC diversity and plasticity

The group of cells called SMCs (reviewed in Owens et al. 2004 (Owens, Kumar et al. 2004)) is heterogeneous in many respects. SMCs taken from one part of the body do often not resemble SMCs from another part of the body – neither in developmental origin nor how they function physiologically (Topouzis and Majesky 1996), (Frid, Aldashev et al. 1997). A SMC in the aorta is for example not necessarily equivalent to a SMC in the bladder. SMCs in pathological situations are furthermore often not equivalent to SMCs in unaffected tissues.

Fully differentiated SMCs are elongated and fusiform – i.e. they are largest at their midpoints and narrow towards their ends. Moreover, they display a contractile apparatus and express SMC marker genes. Unlike for example skeletal and cardiac muscles that are terminally differentiated, SMCs retain a considerable plasticity (Owens, Kumar et al. 2004). SMCs can be more or less matured and display several states of differentiation. SMCs in adult vessels can undergo profound but reversible phenotypic modulations due to environmental changes. Thanks to modulation, they can take part in vascular repair. This plasticity could be regarded as a survival advantage that enables us to withstand changes in the circulatory system. Modulation/switching of the SMC phenotype is however also believed to contribute to the pathophysiology in a number of major diseases in humans like atherosclerosis, cancer, hypertension and asthma.

2.1.2.3. SMC marker genes

In terms of genetic expression profiles, fully differentiated and mature SMCs typically express SMC marker genes. The combination of proteins specific for SMCs is necessary for proper vessel structure and contractile function. The first SMC marker identified was the smooth muscle α-actin (SM α-actin) (Acta2). It is the most commonly used marker for vascular SMCs and a smooth muscle isoform of the contractile apparatus protein actin (Gabbiani, Schmid et al. 1981). It has been used as SMC marker in some of the works presented here. The expression of SM α-actin is however not specific for vascular SMC; SM α-actin is expressed in multiple embryonic cell types, including cardiac and skeletal myoblasts as well as primordial SMCs (Ruzicka and Schwartz 1988), (Sawtell and Lessard 1989). SM α-actin expression alone is therefore not sufficient for identification of primordial vascular SMCs. In a study by Miano and co-workers, the expression of another marker, smooth muscle myosin heavy chain (SM-MHC) was investigated (Miano, Cserjesi et al. 1994). In situ hybridization of staged mouse embryos was used. SM-MHC transcripts were first revealed in the developing aorta at E10.5. No SM-MHC transcripts were observed in the developing brain, heart, or skeletal muscle except for within blood vessels. SM-MHC appears to be a distinct marker for vascular SMCs but its expression is not detected until well after the initial primitive vessel has been

established (E10.5). Other intracellular markers suggested to define SMC characteristics are h1-calponin and SM22α (Duband, Gimona et al. 1993). Calponin is a myofibrillar thin filament of the contractile apparatus. It is an actin-binding protein found in SMCs of adult animals (Winder and Walsh 1993). During development calponin is expressed throughout the developing cardiac tube as well as in differentiating SMCs (Miano and Olson 1996). In the developing dorsal aorta and cardiac outflow tract calponin expression is detectable at E9.5 (Samaha, Ip et al. 1996). SM22α is a cytoskeletal protein structurally related to calponin. It has been shown to be expressed specifically in adult SMCs and transiently in embryonic cardiac and skeletal muscle (Li, Miano et al. 1996). In embryonic tissues from mice, SM22α mRNA can be detected in vascular SMCs from E9.5 (Li, Miano et al. 1996). In the works presented here, SM22α-lacZ mice (Ref. Zhang, Kim, Helmke 2001,Mol. Cell Biol. 21, 1336-1344) were used to detect early signs of vascular SMC differentiation. Intermediate filaments expressed by vascular SMCs are desmin and vimentin. None of them is a specific SMC marker but early markers of muscle tissue in general. Vimentin is expressed in muscle and nonmuscle cells early in the developing embryo, before the emergence of a vascular system (Franke, Grund et al. 1982). Desmin is found in smooth muscle as well as in skeletal and cardiac muscle (Junqueira 1995). Expression of desmin in the developing dorsal aortae in mice starts at E9.5. This expression seems transient; at E14.5 little or no aortic expression remains (Raguz, Hobbs et al. 1998). Caldesmon is a calmodulin and actin-binding protein found in smooth muscle and nonmuscle cells (Sobue, Muramoto et al. 1981). The h-caldesmon isoform can be found in differentiated smooth muscle tissues while the l-caldesmon isoform is widely distributed in nonmuscle tissues and cells (Ueki, Sobue et al. 1987). In the developing embryo, h-caldesmon has been considered to be a late differentiation determinant in smooth muscle; in general it appears after markers like desmin and SM α-actin (Duband, Gimona et al. 1993). The intracellular protein metavinculin is a smooth muscle-variant of vinculin. It has been shown to be localized at junction sites where cell membranes and microfilament bundles join in smooth and cardiac muscle (Geiger, Tokuyasu et al. 1980), (Belkin, Ornatsky et al. 1988). It has also been found in cultured myofibroblast cells (Ehler, Babiychuk et al. 1996). Telokin has been shown to be involved in SMC relaxation in vitro (Wu, Haystead et al. 1998). In embryonic mouse tissues telokin expression is restricted to SMCs and

can be detected from E11.5 (Herring, Lyons et al. 2001). Smoothelin is a cytoskeleton-associated protein. It appears with two iso-forms (type A and B) that are expressed in visceral and vascular SMCs respectively (van der Loop, Schaart et al. 1996), (Rensen, Merkx et al. 2000). Smoothelin is considered to be a late differentiation marker; it appears later than SM α-actin, desmin, and SM-MHC. Its expression has been suggested to be restricted to contracting SMCs (van der Loop, Gabbiani et al. 1997).

2.1.3. Blood vessel development

2.1.3.1. Vasculogenesis and angiogenesis

The hemangioblast is a mesoderm-derived common progenitor to hematopoietic stem cells and angioblasts (Pardanaud, Yassine et al. 1989). In the process of vasculogenesis, endothelial cells are differentiated out of angioblasts to form a primitive endothelial cell tube. The differentiation of angioblasts into endothelial cell tubes requires vascular endothelial growth factor (VEGF) (Ferrara, Carver-Moore et al. 1996). The hereby formed primary capillary plexus of endothelial cell tubes will be remodeled and further refined to become capillaries, arteries or veins in a process called angiogenesis. Vasculogenesis and angiogenesis follow different developmental principles. In the process of patterned vasculogenesis, vessel morphogenesis is controlled by uniform gene expression programs. As a result, vessels formed by vasculogenesis (dorsal aorta, aortic arches and facial vessels) always end up in the same location in different individuals. The process of angiogenesis on the other hand includes no differentiation step of endothelial cells; in angiogenesis, endothelial cells are recruited from nearby vessels by sprouting. VEGF mediates proliferation of the newly formed capillaries and sprouting of new vessels by loosening of cell contacts and degradation of the extracellular matrix at certain points. The primary endothelial cell tube will be invested by contractile support cells. Recruitment of vascular SMCs and pericytes through co-migration along angiogenic sprouts needs signaling of platelet derived growth factor (PDGF) by the endothelial cell population (Lindahl, Johansson et al. 1997), (Hellstrom, Kalen et al. 1999).

2.1.3.2. Vascular SMC origin

The two lateral aortae are located between the neural tube and the future gut. They are formed at E8.0 (Kaufman and Bard 1999). The formation of a midline dorsal aorta at E11.0 includes a fusion of the two lateral aortae. The fusion process has been compared to a zipper, placed in an anterior-posterior direction and gradually closing. Proper fusion of the lateral aortae requires sonic hedgehog signaling (Nagase, Nagase et al. 2006), (Kolesova, Roelink et al. 2008). Investment of contractile support cells starts during the fusion process of the dorsal aortae and continues through development. Cells expressing SMC differentiation markers are first seen on the ventral side of the fusing endothelial tubes and between these structures (Hungerford, Owens et al. 1996).

As discussed above, the population of vascular SMCs can be more or less differentiated and display dissimilarities in smooth muscle marker gene expression. In addition to phenotypic heterogeneity, vascular SMCs also have many possible origins; they can arise from several different independent cell lineages during development (Gittenberger-de Groot, DeRuiter et al. 1999), (Majesky 2007). The origin of SMCs in the descending mouse aorta has not been convincingly determined.The developmental origin of SMCs may be important, both in normal physiology and in disease. In a paper from 1996, dramatic lineage-dependent differences of SMCs in their responses to transforming growth factor β1 (TGF-β1) were reported. These experiments were performed in cultured cells from chicken. SMCs of ectodermal origin were shown to be growth stimulated by the factor TGF-β1 while SMCs of mesodermal origin were inhibited in growth by the same factor. Analogously, it was also shown that mesoderm-derived and ectoderm-derived cells exposed slightly different TGF-β receptors (Topouzis and Majesky 1996). The origin of a vascular SMC might be dependent on where the cell is located in the body and whether it resides in a normal healthy vessel or in a vessel undergoing pathological changes. Experimental tools like chick-quail chimaeras, retroviral markers and certain Cre-recombinase mouse strains have made it possible to perform detailed lineage tracing experiments. Aortic SMCs have been shown to originate from several different sources; the cellular origin of

vascular SMCs is complex. As noted earlier, both ectodermal and mesodermal progenitors can give rise to these cells.

2.1.3.2.1. Neural crest contribution

Studies of embryonic chick-quail chimeras in 1975 revealed that ectodermal cells could give rise to vascular SMCs. It was shown that in arteries derived from the branchial arches, SMCs were made up of mesectodermal cells that had emigrated from the dorsal side of the neural tube. These cardiac neural crest cells were accumulated in the walls of branchial arch arteries. In contrast, endothelial cells in these arteries were of mesodermal origin (Le Lievre and Le Douarin 1975). In another study, also performed in chick-quail chimeras, it was found that migrated neural crest cells mediated septation of the aorta and pulmonary trunk in the cardiac outflow tract. If the population of neural crest cells were removed before migration had occurred, normal patterning of the aorticopulmonary septum was disturbed. As a result of such removal, common arterial outflow channels or transposition of the great vessels were seen (Kirby, Gale et al. 1983). What was shown in chick-quail chimaeras is valid for many different vertebrate species. By crossing transgenic mouse strain Wnt1-Cre and the floxed stop ROSA26 reporter (R26R) mouse, Jiang et al. labeled the neural crest cell population in mice at the time of its formation and later traced its contribution to the vasculature (Jiang, Rowitch et al. 2000). In mice, neural crest cells contribute to the formation of the ascending aorta, the aortic arch and aortic arch arteries, pulmonary artery and ductus arteriosus (Creazzo, Godt et al. 1998), (Gittenberger-de Groot, DeRuiter et al. 1999), (Jiang, Rowitch et al. 2000). Neural crest cells also substantially contribute to the adult atrioventricular and semilunar valves as well as to the cardiac conduction system (Nakamura, Colbert et al. 2006). The capacity of neural crest cells to contribute to mesenchymal derivatives in vessel formation extends to the level of the 5th somite in quail and chicken (Le Lievre and Le Douarin 1975) and to ligamentum arteriosus in mice (Jiang, Rowitch et al. 2000). In addition to neural crest cells emigrating from the dorsal side of the neural tube, another neural tube-derived emigrating cell population has been suggested to give rise to vascular SMCs. These ventrally emigrating neural tube (VENT) cells from the ventral part of the hindbrain would contribute to SMCs in the great vessels and the coronary vessels in chicken (Ali, Farooqui et al. 2003). The existence of such cell population and its importance in

cardiovascular development has however been questioned (Boot, Gittenberger-de Groot et al. 2003).

2.1.3.2.2. Proepicardium contribution

The epicardium is the inner mesothelial layer of the pericardium, the sac of fibrous tissue that encloses the heart. The epicardial layer is formed from the proepicardial organ which is a transient primordial population of cells developed from septum transversum mesenchyme. The proepicardial organ also gives rise to SMCs of the coronary vessels (Gittenberger-de Groot, Vrancken Peeters et al. 1998), (Mikawa and Gourdie 1996). During the formation of the epicardial layer over the heart surface, precursors of the coronary arteries simultaneously migrate from the proepicardium over the heart. Coronary vessels are formed by vasculogenesis. They will hereby form by in situ differentiation from migrating precursor cells and not as outgrowths from the aorta (Mikawa and Fischman 1992), (Bogers, Gittenberger-de Groot et al. 1989). The SMC lineage, as well as the endothelial cell lineage in coronary vessels are established prior to the migration, in the proepicardial organ (Mikawa and Gourdie 1996).

2.1.3.2.3. Mesothelium contribution

Mesothelium is the mesoderm-derived layer of cells covering internal organs (visceral mesothelium) or body cavities (parietal mesothelium). The pericardium, pleura and peritoneum are covered by mesothelium. It is also found in the subdural compartment. Most mesothelial cells express the transcription factor Wilms' Tumor-1 (WT1) (Armstrong, Pritchard-Jones et al. 1993), (Moore, Schedl et al. 1998), (Moore, McInnes et al. 1999). In a study by Wilm et al. WT1-Cre mice were crossed with floxed stop ROSA26 mice. Cells of mesothelial origin were hereby labeled and their contributions to the vasculature were traced. It was found that in the major blood vessels of mesenteries and gut, SMCs were derived from serosal mesothelium, while SMCs within the gut wall itself had another origin (Wilm, Ipenberg et al. 2005).

2.1.3.2.4. Secondary heart field contribution

The heart arterial pole is a region in the cardiac outflow tract where the aorta and pulmonary trunk leave ventricular myocardium as distinguishable structures. Secondary heart field is the source of progenitor cells giving rise to the myocardium and SMCs at the base of the aorta and pulmonary trunk in chicken (Waldo, Hutson et al. 2005). In mice it has been shown that secondary heart field-derived cells contribute to the right ventricular infundibulum, pulmonary trunk and pulmonary valves (Maeda, Yamagishi et al. 2006). The arterial pole is a region where several different developmental origins are joined. The seam between myocardium and secondary heart field-derived SMCs and the seam between secondary heart field-derived SMC and neural crest-derived SMCs respectively, are predilection sites of aortic dissections in certain syndromes (Waldo, Hutson et al. 2005).

2.1.3.2.5. Somite contribution

Following gastrulation, the paraxial mesoderm will appear as elongated structures on either side of the embryo, lateral to the neural tube and medial to the intermediate and lateral plate mesoderm. Paraxial mesoderm is divided into blocks or segments called somites. Segmentation of the paraxial mesoderm starts in the rostral region of the embryo and proceeds caudally during somite formation. The somites are transitional structures that will divide into an outer part called dermomyotome and an inner part called sclerotome. The dermomyotome will be further separated into myotome (giving rise to skeletal muscle) and dermatome (developing to dermis). The sclerotomal cells will migrate medially to surround the neural tube and notochord and eventually give rise to vertebrae and ribs (Kaufman and Bard 1999). Recently several papers have shown a somite contribution to aortic SMCs in the avian embryo. Pouget et al. used transplantation of chick-quail segmental plate grafts, i.e. parts of paraxial mesoderm prior to segmentation. In the chimaeras hereby formed, they could demonstrate a somite contribution to endothelial and vascular SMCs in the aorta (Pouget, Gautier et al. 2006). Esner et al. could show that during mouse development some cells of the dorsal aorta and some cells of the myotome seem to share a common progenitor. From clonal retrospective analyses and SM α-actin staining pattern they

suggested that some aortic SMCs arise from the myotome (Esner, Meilhac et al. 2006). In order to determine what part of the somites that is able to provide vascular SMC precursors, Wiegreffe et al. performed chick-quail transplantations of dermomyotome and sclerotome respectively. In contrast to what Esner et al had found, it was demonstrated that it is the *sclerotome* compartment of somitic mesoderm that will give rise to vascular SMCs, not the dermomyotome (Wiegreffe, Christ et al. 2007). A follow-up study by Pouget et al. led to the same conclusion. It was furthermore shown that in addition to the aorta, vascular SMCs of the avian body wall and limbs originate from the somites (Pouget, Pottin et al. 2008).

2.1.3.2.6. Lateral plate mesoderm contribution

As discussed earlier, the descending aorta is developed from the two fused dorsal aortae. Initially the vessel consists of a single layered endothelial cell tube that is gradually invested by vascular SMCs (Takahashi, Imanaka et al. 1996). It has generally been assumed that splanchnic lateral plate mesoderm surrounding the endothelial cell tubes will contribute to vascular SMCs. Clear experimental evidence for this theory has however been lacking (Gittenberger-de Groot, DeRuiter et al. 1999). In some early studies, conclusions about cellular origin were drawn from expression patterns of certain SMC markers. In an article from 1992, it was reported that in amphibians, SM α-actin functions as a marker of ventrolateral mesoderm and its derivatives. Since vascular SMCs were marked by SM α-actin as well, it was concluded that vascular SMCs were of lateral plate mesoderm origin (Saint-Jeannet, Levi et al. 1992). In a similar study, it was found that SM22α was expressed in lateral plate mesoderm as well as in vascular SMCs. The authors suggested that vascular SMCs were of lateral plate mesoderm origin (Oka, Shiojima et al. 2000). In the avian embryo, cells expressing SMC marker genes are first detectable in the ventral aspect of the aortic vessel wall (Hungerford, Owens et al. 1996). The first cells that express SMC markers are furthermore located immediately adjacent to the endothelium. It was assumed that the mesenchyme surrounding the endothelium contains vascular SMC progenitors that are induced to become SMCs by actions of the endothelium (Hungerford, Owens et al. 1996).

2.1.3.2.6.1. Ventral induction/dorsal migration of SMC

The induction pathways for de novo formation of vascular SMCs are not yet known, as will be discussed. The expression of SMC contractile proteins (e.g. SM α-actin) is however first detectable in the ventral part of the aorta (Hungerford, Owens et al. 1996). Hungerford et al. also concluded that mesodermal cells were not recruited to the aorta equally from all directions, but in a ventral-to-dorsal manner (Hungerford, Owens et al. 1996). In mice, the first signs of SMC differentiation are visible at E9.0. At this point, the mesenchyme surrounding the vessel at the dorsal side is loosely organized with rather few cells, sparsely distributed. This led to the conclusion that vascular SMC induction took place in the mesenchyme ventral to the aorta and that induced SMCs later migrated to populate dorsal positions in the vessel wall (Figure 1).

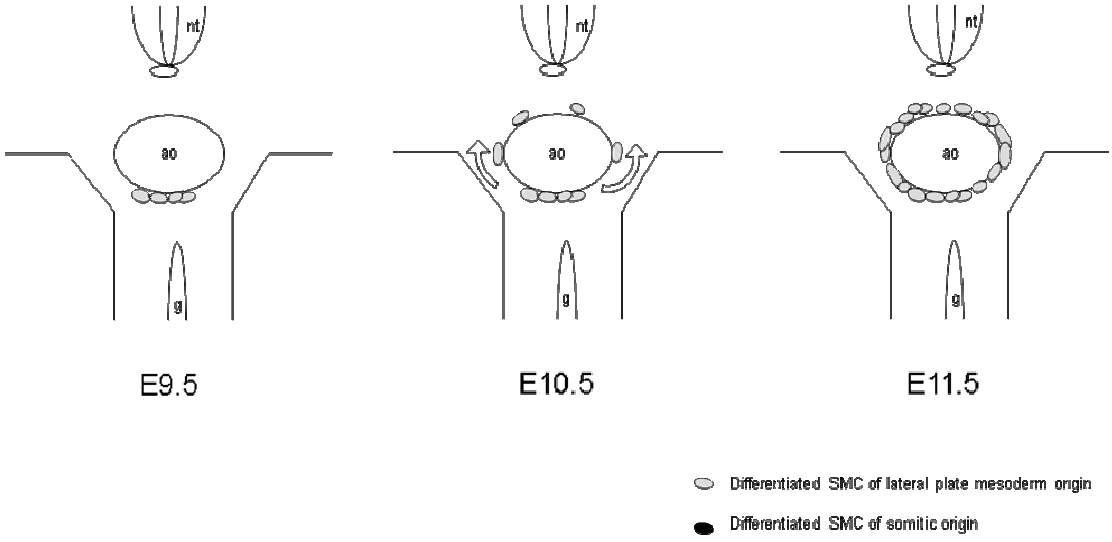

Figure 1. The hypothesis of a ventral-to-dorsal migration of induced SMCs in the aortic vessel wall. According to this theory, all aortic SMCs were of lateral plate mesoderm origin. (nt=neural tube, ao=aorta, g=gut)

Although no experimental evidence for such migration exists it has been a dominant theory. In a paper by Liu et al. 2000, Edg1 knockout mice were reported to lack both vascular SMCs in the dorsal sector of the aorta and pericytes in the brain. In addition, one could see a migration defect in vitro of cultured Edg-$1^{-/-}$ mouse embryonic fibroblast (MEF) cells. It was concluded by Liu and co-workers that the lack of SMCs in the dorsal part of the vessel in Edg1 knockout mice was due to defect cell migration from the ventral wall of the aorta (Liu, Wada et al. 2000).

2.1.3.2.7. Multipotential cells contribution

2.1.3.2.7.1. Mesangioblasts

As discussed earlier, the hemangioblast is a cell type able to give rise to both hematopoietic stem cells and angioblasts (Pardanaud, Yassine et al. 1989). Cossu et al defined another multipotential cell type: the "meso-angioblast" or "mesangioblast" that was isolated from E9.5 murine aortas (Cossu and Bianco 2003). In addition to giving rise to angioblasts and endothelial cells, the mesangioblast also had myogenic potential. It differentiated into skeletal muscle, smooth muscle and other mesenchymal cells in vitro (De Angelis, Berghella et al. 1999). Experiments in chicken with grafted mesangioblasts furthermore showed that these cells were able to travel with the bloodstream and could be integrated into a number of different mesodermal tissues: blood, cartilage, bone, cardiac muscle, skeletal muscle and smooth muscle. They were also integrated into arteries, where they expressed desmin and SM α-actin (Minasi, Riminucci et al. 2002).

2.1.3.2.7.2. Progenitor/stem cells

In vitro studies have shown that mouse embryonic stem (ES) cells that express VEGF receptor 2 (flk+ cells) can be induced to differentiate into SMCs or endothelial cells, depending on culture medium. Addition of platelet-derived growth factor B (PDGF-B) to the medium promoted a SMC phenotype and made the cells express SM α-actin (Yamashita, Itoh et al. 2000). It was suggested that flk1+ cells were able to act as vascular progenitor cells. Circulating progenitor cells and stem cells have been reported to be involved in angiogenesis or vascular repair as a response to injury. A

certain population of progenitor cells named “side population cells” was found in the tunica media of adult mice aortas. When these cells were exposed to VEGF, they developed an endothelial phenotype and when they were exposed to TGF-β or PDGF-B, they were differentiated into SMCs. It was concluded that the arterial wall in adult mice contained cells with vascular progenitor potential (Sainz, Al Haj Zen et al. 2006). In the adventitia, another SMC progenitor cell population was found (Hu, Zhang et al. 2004). When exposed to PDGF-B, they were differentiated into SMCs and when exposed to VEGF-A, they developed characteristics of endothelial cells. It was suggested that these adventitial cells contributed to the SMCs seen in atherosclerotic lesions. It has furthermore been suggested that SMCs participating in neointima formation may arise from circulating hematopoietic stem cells (Saiura, Sata et al. 2001), (Sata, Saiura et al. 2002).

2.1.3.3. General conditions for differentiation

Cells and organs acquire tissue-specific properties during development. The formation of differentiated cells and tissues out of the three germ layers is a complicated process not fully understood. In a process called induction or proximate interaction, one group of cells change the behavior of an adjacent set of cells of originally different history and other properties. The adjacent cells may change shape, mitotic rate or fate as a consequence of the induction. A necessary condition for induction to occur is the ability to respond to an inductive signal. This ability is called competence. Competence is an actively acquired condition; cells gain competence after being influenced by special competence factors. Correspondingly, a cell can lose its competence during development (Gilbert 2000).

2.1.3.4. Vascular SMC induction

How mesenchymal cells obtain the differentiated state of a vascular SMC remains an unanswered question in vascular biology. In mice, SM α-actin-positive SMCs first appear at E9.0. It has been suggested that undifferentiated mesenchymal cells prior to this event have received a signal of induction. From where such differentiating signal would be sent or what factor it would be has not been elucidated. One suggestion is

that SMC differentiation is initiated when a local induction signal is provided by the endothelium (Takahashi, Imanaka et al. 1996). Zerwes et al. could demonstrate that cultured bovine endothelial cells stimulate migration of SMCs and that the endothelial cells' chemotactic activity can be inhibited by PDGF antibodies (Zerwes and Risau 1987). Based on the position of the first SMCs seen in the aorta during development, Hungerford et al. hypothesized that an inductive signal was released from ventral endothelium or from some other ventral structure, like the endoderm (Hungerford, Owens et al. 1996). The endoderm has previously been shown to act as an inducer of mesodermal tissues (Jacobson and Sater 1988). An alternative hypothesis was also presented by Hungerford et al. in which all endothelial cells around the circumference of the aorta were equally good at recruiting SMC progenitors. The fact that SMCs are first detected in the ventral aspect of the vessel was explained by a hypothesized inhibiting signal from the notochord, which delayed SMC induction and recruitment in the dorsal area. It was speculated that this inhibiting factor would be sonic hedgehog (Hungerford, Owens et al. 1996). In a study by Hirschi and co-workers, so called 10T1/2 cells were used as presumptive mural cell precursors. In co-culture with endothelial cells, 10T1/2 cells changed their appearance from flat polygonal to elongated spindle-shaped and they increased their expression of SM α-actin, smooth muscle myosin, calponin and SM22α. It was suggested that the 10T1/2 cells had differentiated towards a SMC fate (Hirschi, Rohovsky et al. 1998). It was furthermore suggested that the signal inducing differentiation was TGF-β and that migration was induced by PDGF-B. The importance of TGF-β has also been pointed out by other groups (Ross 1993), (Adam, Regan et al. 2000), (Hautmann, Madsen et al. 1997). Chen at al. demonstrated that a neural crest stem cell line was induced to display markers and characteristics of vascular SMCs after exposure to TGF-β (Chen and Lechleider 2004). Furthermore, it has been suggested that mechanical forces would induce a more differentiated SMC phenotype. In a study by Reusch et al. neonatal rat vascular SMCs were cultured on silicone tubes and exposed to mechanical stress. It was found that the treatment altered myosin isoform expression towards that found in a more differentiated state of SMC (Reusch, Wagdy et al. 1996).

As discussed earlier, a proper investment of vascular SMCs to the endothelial cell tube during development requires PDGF-B. Lindahl et al. found that PDGF-B

released from the endothelium was important for migration and proliferation of induced vascular SMCs (Lindahl, Johansson et al. 1997). Expansion of the induced pool of SMCs has been suggested to be PDGF-B-dependent while SMC induction would be PDGF-B-independent (Hellstrom, Kalen et al. 1999). PDGF-B has been shown to influence TGF-β expression which could link PDGF also to SMC induction. In a study by Nishishita et al. it was shown that PDGF-B was able to increase TGF-β expression in cultured cells (Nishishita and Lin 2004). Yamashita et al. used flk1+ cells derived from ES cells to show that an undifferentiated cell line exposed to PDGF-B was induced to differentiate towards mural cells (Yamashita, Itoh et al. 2000). Miyata et al. obtained similar results in another cell line. VEGF promoted differentiation towards endothelial cells and PDGF-B promoted differentiation towards SMCs (Miyata, Iizasa et al. 2005). PDGF-B can however also act as an efficacious and selective negative regulator of SMC differentiation. It has been shown in several papers that SMCs lose their differentiation in the presence of PDGF-B. Corjay et al. demonstrated a drop in SM α-actin mRNA when PDGF was added to rat aortic SMCs. (Corjay, Thompson et al. 1989). Holycross et al. demonstrated that addition of PDGF-B decreased expression of SM-MHC and SM α-tropomyosin dramatically in cultured SMCs (Holycross, Blank et al. 1992). In a study by Wang et al., the role of PDGF-B in serum response factor (SRF)-dependent transcription was studied. It was found that PDGF suppressed the expression of contractile protein genes SM22 and SM α-actin in SMCs by decreasing the association between SRF and myocardin. SRF co-factor Elk-1 was instead phosphorylated and activated at SRF target sites, resulting in a switch from a muscle-specific transcriptional program towards a growth-regulated transcriptional program (Wang, Wang et al. 2004).

2.1.3.5. Alternative vascular SMC development

2.1.3.5.1. Endothelial-to-SMC transdifferentiation

It has been suggested that endothelial cells may transdifferentiate to form vascular SMCs. The endothelial transdifferentiation would appear either from endothelial progenitor cells or from the mature endothelium. This mechanism has been suggested to represent a general pathway for vascular SMC development both in embryonic

vessel development and in atherosclerosis (DeRuiter, Poelmann et al. 1997). As discussed earlier, Yamashita and co-workers could show that a population of mesodermal ES cells expressing the VEGF receptor flk1 could differentiate to endothelial cells as well as to mural cells (Yamashita, Itoh et al. 2000). A common progenitor cell for endothelial and vascular SMCs was suggested, in line with the endothelial transdifferentiation data. In opposition to these data, cell tracing studies of endothelial cells in Tie1-Cre/ROSA26 mice show no contribution of endothelial cells to vascular SMCs (Gustafsson, Brakebusch et al. 2001).

2.2. Transcriptional regulation of SMCs

2.2.1. SMC marker gene promoters contain CArG boxes

Much effort is presently put on mapping common traits of the regulatory sequences in all SMC differentiation marker genes. The most reoccurring DNA sequence motif in promoters and/or intronic sequences of such genes is the CArG box. This cis-regulatory element was first identified in the growth responsive gene c-fos and then named Serum Response Element (SRE) (Treisman 1986). To date about 170 CArG-containing genes have been identified (Miano 2008). Examples include skeletal α-actin, α-myosin heavy chain, β-myosin heavy chain, myosin light chain, SM22α, telokin, troponin, tropomyosin, calponin, atrial natriuretic factor, Sarcoplasmic reticulum Ca^{2+}-ATPase, dystrophin and creatine kinase M (Chai and Tarnawski 2002). The SRE element sequence is not completely alike in all SRF-dependent genes. It has the following consensus sequence: CCTTATATGG, shortly written $CC(A/T)_6GG$ and usually referred to as the CArG element or CArG box. It has been shown that expression of almost all SMC marker genes so far identified are dependent on one or more CArG elements; they have been shown to be necessary for transcription of e.g. SM22 (Li, Liu et al. 1997), SM-MHC (Madsen, Regan et al. 1998), SM α-actin (Mack and Owens 1999), CRP1 (Lilly, Olson et al. 2001) and Telokin (Herring and Smith 1997). However, as mentioned earlier CArG boxes are not restrictively confined to regulate SMC differentiation. Furthermore there are examples of SMC marker genes that are expressed in a CArG box-independent

manner, for example ACLP (Layne, Yet et al. 2002) and HRC (Anderson, Dodou et al. 2004). Studies of site-directed mutagenesis in transgenic mice have shown that mutation of certain CArG motifs will result in differential effects depending on SMC subset. For example Owens and co-workers performed systematic mutations of the three known CArG boxes of the SM-MHC gene (Manabe and Owens 2001). If CArG1 was mutated (this is the most proximal CArG element in the 5'-region of the SM-MHC promoter) all expression in every SMC subtype was abolished. Mutation of the intronic CArG element on the other hand had no effect on SM-MHC expression in muscular arteries, pulmonary airway SMC or gastrointestinal SMC but in large-conduit arteries and in the coronary circulation. It seems like different subsets of SMC will employ different modular regulatory regions for expressing a certain SMC differentiation marker.

2.2.2. CArG boxes are binding sites for SRF

The SRF protein was first discovered and named because of its ability to bind the SRE described above. In humans two mRNA isoforms of SRF have been identified while mouse carry four isoforms (Kemp and Metcalfe 2000). There is a 93% homology in SRF between fruit fly and human (Affolter, Montagne et al. 1994). The SRF protein contains 508 amino acids and has three major domains: i) a CArG box DNA-binding domain; ii) a transactivation domain; and iii) several phosphorylation sites. SRF binds CArG boxes as a dimer. The DNA-binding and dimerization domains are both situated between amino acids 133 and 222 (Norman, Runswick et al. 1988). This 90 amino acid domain can perform DNA-binding, dimerization and interaction with accessory factors. It is a domain highly conserved in eukaryotes. Sometimes it is referred to as the MADS box (MCM-1 (from yeast), Agamous and Deficiens (from plants) and SRF (from animals)) (Shore and Sharrocks 1995). Several SMC-specific marker genes, e.g. SM α-actin and SM-MHC have their transcriptional regulation controlled by SRF (Simonson, Walsh et al. 1995), (Madsen, Hershey et al. 1997). SRF is however expressed in many tissues. It has been discussed how SRF can function as a major transcription factor in SMC differentiation while it is ubiquitously expressed. One explanation emanates from the fact that SRF is highly expressed in SMCs compared with most other tissues (Belaguli, Schildmeyer et al. 1997).

Moreover, CArG elements within SMC-specific genes tend to have substitutions in their motifs that actually lowers SRF-binding affinity (Hautmann, Madsen et al. 1998). Perhaps these two phenomena together serve to create a cell-selectivity by restricting expression of SMC marker genes only to cells that express high levels of SRF. Another explanation to the selectivity would be that SMC-specific genes in their promoters tend to have more than one CArG box. The promoter often does not work unless all elements are intact. The manner these CArG boxes are organized (which is conserved between species) might contribute to SMC-selective expression of CArG-dependent SMC genes. However, spacing between multiple CArG boxes within a promoter offers no explanation to SMC-specific genes with only one CArG box (e.g. Telokin) or in fact no CArG box at all (e.g. Aortic carboxypeptidase-like protein (ACLP)) (Owens, Kumar et al. 2004). In recent years it has been found that the tissue specificity of SRF is also obtained by transcriptional cofactors, like myocardin.

2.2.3. Myocardin is a cofactor for SRF-dependent transcription in SMCs

SRF directs transcriptional programs that regulate SMC development and differentiation. It is a widely expressed transcription factor that requires tissue-specific cofactors for its specificity (Miano, Ramanan et al. 2004). Using a bioinformatics approach, myocardin was discovered to be a transcriptional cofactor of SRF (Wang, Chang et al. 2001). After having formed complexes with SRF, myocardin works as a very potent transcriptional activator for CArG box-dependent promoters (Wang, Chang et al. 2001). Myocardin expression pattern has been determined by in situ hybridization on staged mice embryos. During development, myocardin is expressed in the heart and in vascular and visceral SMCs (Du, Ip et al. 2003). Myocardin knockout mice die at E11.5 from a lack of vascular SMCs (Li, Wang et al. 2003). In adult humans, myocardin is expressed in the heart, aorta and in some SMC-containing tissues: stomach, bladder, small intestine, colon, and uterus (Du, Ip et al. 2003).

Myocardin belongs to the transcription factor family of MRTFs (myocardin-related transcription family) (Parmacek 2007). Other MRTFs are MRTF-A and MRTF-B. All MRTFs use SRF as mediator of their transcriptional activity; if SRF is deleted (e.g. in $SRF^{-/-}$ ES cells), the transcriptional activities of MRTFs are lost (Wang, Li et al.

2002). In contrast to myocardin, MRTF-A and MRTF-B are expressed in many different kinds of embryonic and adult tissues. MRTF-A is co-expressed with myocardin in SMCs (Du, Chen et al. 2004). Myocardin and MRTF-A both have the ability to activate smooth muscle gene expression in cultured mouse embryonic multipotential 10T1/2 cells (Wang, Wang et al. 2003). High MRTF-A expression has also been reported in some mesenchymal cells, some skeletal muscle cells and some epithelial cells (Wang, Li et al. 2002). Fetal expression of MRTF-B is regulated in a cell lineage-restricted pattern. At E 8.5, MRTF-B is expressed in cells forming cardiac neural crest cells, i.e. cells that will give rise to SMCs in the cardiac outflow tract and aortic arch arteries (Li, Zhu et al. 2005). MRTF-B-deficient mice display deformations of aortic arch arteries 3, 4 and 6, in addition to reduced SMC differentiation in the aortic arch arteries and aorticopulmonary septum (Li, Zhu et al. 2005). MRTF-B is also highly expressed in some epithelial cells and intestinal SMCs (Wang, Li et al. 2002).

The myocardin and MRTF proteins contain certain functional regions: one domain responsible for actin-binding, one responsible for SRF-binding and one responsible for dimerization with other MRTF members. Myocardin performs dimerization in vitro and it has been suggested that dimerization is required for maximal activity of myocardin (Wang, Wang et al. 2003). All MRTFs also have a so called SAP-domain (SAF-A/B, Acinus, PIAS). Proteins with SAP-domains have been shown to take part in chromatin remodeling, apoptosis and transcriptional control (Aravind and Koonin 2000). Furthermore, it has been shown that the myocardin gene is able to encode two mRNA splice variants and that two alternative protein isoforms are detected in different tissues. A myocardin of 935 amino acids predominates in cardiac muscle while a 856 amino acids myocardin predominates in smooth muscle (Creemers, Sutherland et al. 2006).

Myocardin has been regarded as a master regulator of smooth muscle gene expression (Wang, Wang et al. 2003). Forced expression of myocardin has been shown to activate expression of SM22α, SM α-actin and calponin-h1 genes in undifferentiated mouse ES cells (Du, Ip et al. 2003). Myocardin is however not sufficient to initiate a complete SMC differentiation program of undifferentiated cells (Yoshida, Kawai-

Kowase et al. 2004). Moreover, the expression pattern of myocardin during development conflict with a possible role of myocardin as an inducer of aortic SMC differentiation. The myocardin gene is expressed abundantly in the primitive mouse heart at E9.5 (Wang, Chang et al. 2001). At E9.5, vascular SMCs are observed in the dorsal aorta. Myocardin mRNA is however not detected in the dorsal aorta until E11.5, indicating that aortic SMC differentiation precede the expression of myocardin (Du, Ip et al. 2003).

2.2.4. Many CArG-dependent SMC marker proteins belong to the protein family of LIM-domains

The LIM-domain is a protein-binding interface that regulates either subcellular localization or biological activity of LIM proteins. Its presence indicates a protein with ability to associate to the actin cytoskeleton or to the transcriptional machinery. LIM-domains have been identified in a number of proteins involved in differentiation and transcriptional control. The DNA sequence that encodes the protein LIM-domain was first discovered in three different genes in the nematode Caenorhabditis elegans. The three genes were LIN-11, Isl1 and MEC-3; LIM is an acronym for these genes (Kadrmas and Beckerle 2004). All LIM-domains have 8 conserved residues at defined intervals containing mostly cysteine and histidine. These conserved residues hold two zinc ions which are essential parts of the LIM-domain zinc finger motif (Michelsen, Schmeichel et al. 1993). To date about 135 LIM-encoding sequences have been identified in the human genome. These sequences take part in about 60 human genes (every LIM protein may contain 1-5 LIM-domains) (Kadrmas and Beckerle 2004). If they are linked to homeodomains the LIM proteins act exclusively in the nucleus as transcription factors (Hobert and Westphal 2000). Because of its structural similarity to some other transcriptional factors, it has been suggested that one of the zinc fingers would have a DNA-binding function (Perez-Alvarado, Miles et al. 1994). Most common however, LIM proteins are situated in the cytoplasm, associated with the actin cytoskeleton. Some LIM-containing proteins shuttle between the nucleus and the cytoplasm and are able to influence gene expression. Relevant examples include: four-and-a-half LIM (FHL) (Muller, Metzger et al. 2002) - and cystein-rich protein (CRP) protein-families (Chang, Belaguli et al. 2003). FHL2 is expressed in heart muscle and is suggested to be a co-factor to SRF and a negative regulator of SRF-

dependent gene expression. CRP1, CRP2 and CRP3 are positive regulators of SRF-dependent gene expression and are selectively expressed in smooth muscle, vascular smooth muscle and heart muscle/skeletal muscle, respectively (Henderson, Macalma et al. 1999), (Jain, Fujita et al. 1996), (Arber, Hunter et al. 1997). A third group of proteins that are known to shuttle between the nucleus and the cytoplasm is the zyxin family. To date it consists of the following proteins: Ajuba, LIMD1, LPP, Migfilin, TRIP6, WTIP and Zyxin. Zyxin is often localized to adhesion plaques in cultured cells, at sites of cell adhesion to the extracellular matrix, at cell-to-cell contacts (Beckerle 1986) and at the leading edge of migrating cells (Golsteyn, Beckerle et al. 1997). Since Zyxin also contains LIM-domains it might be a link between mechanical events and the differentiation state or developmental fate of a cell (Cattaruzza, Lattrich et al. 2004). In chicken, Zyxin has been shown to shuttle between the cytoplasm and the nucleus (Nix and Beckerle 1997). The Zyxin knockout however had no specific phenotype, perhaps due to overlapping functions of LPP and TRIP6 (Hoffman, Nix et al. 2003).

2.2.5. LPP is a LIM-domain protein that can shuttle between the nucleus and the cytoplasm

As the name implies, the gene encoding lipoma preferred partner (LPP) was first found in connection to lipomas. About two thirds of all lipomas involve translocation of chromosome segments 12q13-q15 (High Mobility Group Protein Gene HMGIC) (Sreekantaiah, Leong et al. 1991). Every chromosome could principally be involved in such translocation and receive translocated chromosome fragments. It has however been shown that some translocation partners are preferred; one fourth of all translocations involving 12q13-q15 actually use the gene coding for LPP as translocation partner. LPP is expressed in SMCs (Gorenne, Nakamoto et al. 2003), (Nelander, Mostad et al. 2003). It can also be found in other mature tissues like heart muscle myocytes and skeletal muscle. In these tissues however, LPP is present only in trace amounts and the expression of LPP correlates to the expression levels of SM α-actin. In 1996 it was shown that the LPP protein contains LIM-domains (Petit, Mols et al. 1996). As mentioned before, LPP is also one of the zyxin family members. LPP consists of a 612 amino acid sequence with a proline-rich N-terminal region and three

LIM-domains in the C-terminal region. Its build-up resembles a lot both Zyxin and one other zyxin family member TRIP6. The proline-rich region contains a nuclear export signal (NES). In a transactivation assay LPP was shown to activate transcription. Furthermore, if the sequence coding for NES was removed, an accumulation of LPP in the nucleus was seen (Petit, Fradelizi et al. 2000). LPP is believed to shuttle between the nucleus and the cytoplasm (Petit, Fradelizi et al. 2000). Moreover, it has been reported as a coactivator of the transcription factor polyomavirus enhancer activator 3 (PEA3) (Guo, Sallis et al. 2006). LPP has been shown to be associated to the cytoskeleton at focal adhesions (Gorenne, Nakamoto et al. 2003), (Petit, Fradelizi et al. 2000), (Li, Zhuang et al. 2003). The proline-rich region contains two sites for binding of VAsodilatator-Stimulated Phosphoprotein (VASP) in cell adhesions (Petit, Fradelizi et al. 2000). VASP proteins control the organization of the actin cytoskeleton. At cell adhesions, LPP also interacts with alpha-actinin via a special alpha-actinin-binding site located in the proline-rich N-terminal region. Alpha-actinin is an actin-crosslinking protein enriched at focal adhesion sites and along stress fibers. LPP and Zyxin compete for the same binding site in alpha-actinin and LPP has a lower affinity for alpha-actinin than Zyxin (Li, Zhuang et al. 2003).

2.2.6. Summary of LPP function

We conclude that LPP is selectively expressed in SMCs and in the heart. The protein has nuclear translocation ability and shuttles between the nucleus and the cytoplasm. In the nucleus, LPP has been shown to have a transactivating capability. Furthermore, LPP belongs to the protein family of LIM-domains.

2.3. Vascular disease and SMCs

Several pathologies of the vascular system are believed to be more or less dependent on the SMCs that build up the vessel wall. Atherosclerosis, tumour angiogenesis, diabetic retinopathy, restenosis following angioplasty and hypertension are conditions that have been linked to properties of SMCs. Changes in the density of vascular SMCs or certain switches in the vascular SMC phenotype seem to contribute to a

number of serious conditions. It has been suggested that the likelihood of vascular SMCs to perform changes leading to vascular pathology may depend on their developmental origin. A detailed mapping of the ontogeny of vascular SMC and analysis of the cellular events that lead to SMC induction and differentiation is therefore required to evaluate the importance of certain vascular SMC developmental origins as a susceptibility factor for vascular disease. Such knowledge is also crucial to understand blood vessel morphogenesis in general. It is also important to learn more about the properties and behaviour of SMCs in complicated pathological processes, like atherosclerosis.

2.3.1. Atherosclerosis and SMCs

Clinical manifestations from atherosclerosis are commonly seen in the coronary arteries, in the cerebrovascular system and in the peripheral vessels of the extremities. Atherosclerosis is an inflammatory and fibroproliferative process, characterized by lesion formation and a gradual narrowing of the arterial lumen. A complication of atherosclerosis is the most common cause of death in Western countries (Ross 1995), (Braunwald 1997). Exactly what initiates the atherosclerotic process is still not known. Based on studies in nonhuman primates in the 1970's, it was suggested that atherosclerosis would be a response to endothelial injury (Ross and Glomset 1973). Ross and co-workers showed that arterial lesions similar to those found in human atherosclerotic plaques could be created experimentally in macaques by removing the arterial endothelium with an intravascular balloon catheter (Stemerman and Ross 1972). This "response-to-injury" model of atherosclerosis has been modified since then, e.g. as the importance of inflammation throughout the disease process has been recognized (Ross 1999), (Libby 2002). Furthermore it has been argued that there is no definitive evidence in vivo that endothelial injury is either necessary or sufficient for lesion formation (Williams and Tabas 1995). Rather, it has been shown that the endothelial cell layer in general remains intact throughout most stages of lesion progression (Taylor, Glagov et al. 1989), (Katsuda, Boyd et al. 1992). In humans, only the most complicated ulcerated lesions have lost their endothelial cell layer (Williams and Tabas 1995). Since atherosclerotic lesions seem to appear at certain susceptible "lesion-prone" sites in the vascular system it has been investigated whether endothelial permeability at these locations would differ from the rest of the

endothelium. Schwenke and co-workers injected 125I-tyramine cellobiose-labeled low-density lipoprotein (LDL) into rabbits and studied how the substance was distributed in the aortic vessel wall. There was no differential change in the endothelial permeability between susceptible and resistant sites in this experimental model. The increased levels of LDL recorded at susceptible sites were shown to be effects of reduced efflux of LDL from the cells, combined with a decreased degradation of LDL (Schwenke and Carew 1989). Rather than increased endothelial permeability due to endothelial injury, endothelial *retention* of lipoproteins might be a key event in early atherosclerosis. The extracellular matrix of the subendothelium, particularly proteoglycans, is believed to take part in retention of atherogenic lipoproteins (Srinivasan, Vijayagopal et al. 1986), (Boren, Olin et al. 1998). It has been shown by means of transgenic mice that subendothelial retention of atherogenic lipoproteins is an early step in atherogenesis (Skalen, Gustafsson et al. 2002). The way subendotelial retention of lipoproteins takes part in atherosclerotic pathophysiology is sometimes referred to as the "response-to-retention" model (Tabas, Williams et al. 2007).

Vascular SMCs are major components of the atherosclerotic lesion and the accumulation and behaviour of these cells may be fundamental to the entire process. It has even been suggested that the increase of intimal SMCs and extracellular matrix components would be the earliest phase of lesion development (Ross and Glomset 1973). Today, the numerous SMCs found in atherosclerotic plaques are considered to be a fibrotic reaction to inflammation (Ross 1999). The source of the accumulating SMCs has been debated. SMCs could arise from 1) proliferation of preexisting SMCs of the intima, 2) migration of SMCs from the vessel wall, 3) migration of other cell types from the vessel wall that will develop into SMCs in the intima or 4) endothelial uptake of circulating SMC progenitor cells. One model to explain intimal accumulation of SMCs is by migration of phenotypically changed SMCs from tunica media to tunica intima. SMCs show a great deal of plasticity, as was discussed earlier. A modulation of the SMC phenotype, with a loss of expression of genes associated with the differentiated SMC, seems important in the pathophysiology of atherosclerosis (Campbell and Campbell 1990), (Thyberg, Blomgren et al. 1997). The phenomenon is often referred to as the switch from a "contractile" phenotype to a "synthetic" one (reviewed in (Mahoney and Schwartz 2005)). In the developing vessel

SMCs are regarded as immature and synthetic. They have high rates of proliferation, migration and production of extracellular matrix components. In the mature vessel on the other hand, SMCs are in general less proliferative and less synthetic. A phenotypic switch including migration, loss of contractility, abnormal proliferation and matrix secretion gives a "synthetic" phenotype. SMCs from areas of intimal thickening feature a synthetic phenotype; they have a more rounded shape and larger amounts of rough endoplasmic reticulum and cytoplasmic organelles compared with normal medial vascular SMCs (Mosse, Campbell et al. 1985), (Takaichi, Yutani et al. 1993). They also synthesize more extracellular matrix components, have a higher proliferative rate and migrate more easily.

It has been suggested that atherosclerotic lesions display an expansion of cells according to the "monoclonal hypothesis". Benditt and Benditt used X chromosome inactivation analysis to show that an atherosclerotic plaque 0.5 cm or greater in diameter contained a monoclonal population of cells. Their interpretation was that the atherosclerotic plaque was a result of a somatic mutation in a single cell and that the likely causes of such mutation would be chemical agents or viruses (Benditt and Benditt 1973). The mutation would, according to this theory, cause a benign neoplasm similar to what is seen in uterine leiomyomas (Benditt 1974). To regard the atherosclerotic plaque as a neoplasm is not consistent with the common view that SMCs appear in the plaque as a fibrotic reaction to inflammation. Other studies have confirmed however that atherosclerotic plaques do carry a monoclonal population of cells (Pearson, Dillman et al. 1978), (Pearson, Dillman et al. 1987), (Murry, Gipaya et al. 1997), (Chung, Schwartz et al. 1998) and that this monoclonal cell population is composed of SMCs (Murry, Gipaya et al. 1997). Plaque T-cells have been shown to be polyclonal as a comparison (Stemme, Rymo et al. 1991). The SMC monoclonality is present in aortic and coronary plaques as well as in diffuse intimal thickening (Murry, Gipaya et al. 1997). One should however perhaps not regard plaque monoclonality as a true clonal response to injury or a neoplasm. In fact, even SMCs from normal arteries can show monoclonal characteristics, which provides evidence that plaque monoclonality instead may result from expansion of pre-existing (developmental) clones of cells (Murry, Gipaya et al. 1997), (Chung, Schwartz et al. 1998). By studying the presence of proliferating cell nuclear antigen (PCNA), a protein involved in the cell cycle, it has furthermore been shown that SMC replication

levels in general are low in atherosclerotic plaques/restenosis following angioplasty (Gordon, Reidy et al. 1990), (Katsuda, Coltrera et al. 1993), (O'Brien, Alpers et al. 1993), (Rekhter and Gordon 1994). This would suggest that clone formation precedes plaque formation and that the pre-existing (developmental) clones are large.

3. Present investigation

3.1. Objectives

The complex process of building a multilayered vessel wall out of an endothelial cell tube has not been thoroughly investigated. In the work presented here, the aim was (Paper I) to perform a morphological survey of aorta development by tracing the developmental origin(s) of vascular SMCs in the descending aorta. Another aim of this thesis was (Paper II) to learn more about the SMC marker lipoma preferred partner (LPP) and its transcriptional regulation. Finally, the aim was (Paper III) to study the proposed role of platelet-derived growth factor B (PDGF-B) and angiotensin II (Ang II) in the development of neointimal hyperplasia after interruption of carotid blood flow.

3.2. Project description

A detailed morphological survey on dorsal aorta development at E9.5-E15.5, P2 and in adult mice was performed. By recombination-based cell lineage tracing experiments, including HoxB6-Cre/R26R and Meox1-Cre/R26R transgenic mice, the developmental origin of vascular SMCs was determined in the descending aorta and its major branches.

The transcriptional regulation of SMC marker gene LPP was studied by bioinformatics as well as by in vitro and in vivo experiments. An alternative transcription start site for LPP was found and functionally tested. The in vitro studies included mRNA expression analyses on different ES cells, electrophoretic mobility shift assays, chromatin immunoprecipitation and luciferase reporter experiments. In vivo studies included mRNA expression analyses on tissues from conditional SRF knockout mice. In order to elucidate the in vivo functions of LPP, an LPP overexpressing transgenic mouse model was furthermore established. The human LPP

gene was hereby put under the influence of the cardiac-α-MHC promoter. However, these mice failed to give offspring carrying the genetic manipulation.

A carotid artery ligation model was used to study neointima formation. Mice were exposed to left carotid artery ligation and thereafter divided in different groups depending on their further treatment. Some had PDGF-receptor inhibitor injections, some had Ang II infusions and some had both treatments. There were also untreated control mice. The mice used for the ligations were either wild type mice or PDGF-B retention motif knockout mice. In total eight different groups and treatments after vessel ligation were validated regarding the possible effect on neointima formation.

3.3. Methodological considerations

3.3.1. Cre-loxP

Gene targeting techniques are based on homologous recombination. Homologous DNA sequences are hereby exchanged in a site-specific manner. The exchange can be "conditional", i.e. restricted to time in development (temporal) or to a certain location (spatial) in the animal. The Cre-loxP system (causes recombination in locus of (x)crossing over of the bacteriophage P1 genome) is one instrument for conditional gene targeting (Hoess, Ziese et al. 1982). The mice used in the study reported here were HoxB6-Cre or Meox1-Cre mice respectively, crossed with ROSA26R mice. The Cre protein has the ability to catalyze recombination between loxP sites. Genetic material that is to be recombined must therefore be flanked by loxP sites ("floxed"). The loxP sites are recognition sites (34 bp long) with palindromic repeats of 13 bp on the ends and a core sequence of 8 bp. It is in the core sequence that the Cre-mediated strand cleavage occurs, followed by exchange between loxP sites. The conditional properties of the Cre-loxP system reside in the control of Cre recombinase expression. The Cre enzyme will be present only during a chosen time period or in a specific tissue, depending on the promoter. In the study reported here, Cre was expressed in a HoxB6 or Meox1-specific manner (Lowe, Yamada et al. 2000), (Jukkola, Trokovic et al. 2005). The Hox-genes are present in all animals and regulate anterior-posterior

segment identity within a body. In mice, HoxB6 is expressed in all tissues originating from posterior parts of the lateral plate mesoderm. Expression within the embryo starts at E8.5 and should remain hereafter (Lowe, Yamada et al. 2000). The Meox1 gene is a homeobox-gene expressed in the unsegmented paraxial mesoderm and later in differentiating somites (Candia, Hu et al. 1992). It is believed that Meox1 is necessary for somite segmentation and for chondrogenic and myogenic differentiation (Mankoo, Skuntz et al. 2003). A reporter gene that labels the Cre-recombinase-activated lineages should have the following properties: under the influence of a ubiquitously expressed promoter, a coding region of a reporter gene is placed. The reporter gene is preceded by a floxed STOP sequence that will be excised by the actions of Cre. We have used a reporter mouse with a conditional lacZ reporter construct in the ROSA26 locus (Soriano 1999).

The chosen cell lineage tracing experiments resulted in a clear answer as to whether aortic SMCs are lateral plate mesoderm-derived or paraxial mesoderm-derived. Other possible approaches could have included chicken-quail hybrids or injection of paint in single cells to enable cell lineage tracing. These techniques would require a change of model organism. We wanted to perform the experiments in a mammal system.

3.4. Results and Discussion

3.4.1. Paper I

The developmental origin of vascular SMCs is complex; several different cell lineages contribute independently (reviewed in (Gittenberger-de Groot, DeRuiter et al. 1999), (Majesky 2007)). Based on the expression pattern of early SMC markers, aortic SMCs have been proposed to derive from splanchnic lateral plate mesoderm that line the endothelium (Hungerford, Owens et al. 1996), (Takahashi, Imanaka et al. 1996). SMC markers in the developing vasculature seem to appear first on the ventral side of the vessel. Based on this fact and the reported finding that Edg1 knockout mice lacked SMC on the dorsal side of the vessel (Liu, Wada et al. 2000) it was assumed that SMC differentiation was induced on the ventral side and that cells later

migrate to dorsal positions. Further experimental evidence for this theory has however been lacking.

We performed cell lineage tracing experiments in mice. By the use of the transgenic mice strains HoxB6-Cre (Lowe, Yamada et al. 2000), Meox1-Cre (Jukkola, Trokovic et al. 2005) and floxed stop ROSA26 reporter mice (Soriano 1999) it was possible to follow cell fates of lateral plate mesoderm and paraxial mesoderm, respectively. Contrary to what has been previously reported by others, our results showed no migration of vascular SMC progenitor cells from ventral to dorsal sides of the vessel during aorta development. SM22α is one of the earliest SMC markers known during development (Li, Miano et al. 1996). It was therefore used to study early signs of SMC differentiation. We used SM22α-lacZ mice (Zhang, Kim et al. 2001) and detected SM22α-driven lacZ expression in cross-sectioned embryos. Cells expressing SM22α on the ventral side of the vessel at E9.5 were shown to have their origin in lateral plate mesoderm. Later on, these cells were replaced by cells of paraxial mesoderm origin. In the adult mouse all vascular SMCs in the aorta were shown to derive from paraxial mesoderm and not from lateral plate mesoderm.

Reporter-activation by HoxB6-Cre in lateral plate mesoderm-derived cells precedes the induction of vascular SMC differentiation. Our chosen approach works for cell lineage tracing.

The expression pattern of the HoxB6-Cre-activated reporter gene R26 was studied in detail in mouse embryos E8.5-9.0. Reporter expression could be detected in somatic and splanchnic lateral plate mesoderm posterior of the 12th somite. At the very posterior end of the embryo, reporter expression was more widespread and could be seen also in paraxial mesoderm and in the neural plate. In gut epithelium and in extra-embryonic mesoderm, an ectopic reporter expression was seen. In order to detect the earliest stages of SMC differentiation SM22α-lacZ mice were used. At E8.5 no SM22α-lacZ staining (that would indicate initiated SMC differentiation) was detected in regions that stained positive for the HoxB6-Cre-activated R26 reporter at this stage. The first SM22α-lacZ-expressing cells seen were detected at E9.0, in splanchnic mesoderm, adjacent to the aorta and in the space between the initially paired dorsal

aortas. HoxB6-Cre hereby activated the reporter gene in all lateral plate mesoderm-derived cells before the first detectable signs of SMC differentiation.

Lateral plate mesoderm-derived SMCs in the descending aorta are confined to the ventral vessel wall and at E11.5 they are replaced by cells of another origin.

Staining patterns of stage-matched E9.5 HoxB6-Cre/R26R and SM22α-lacZ mouse embryos were compared. In the forelimb bud region, signs of SMC differentiation were discovered in a layer of cells around the circumference of the dorsal aorta. In more posterior parts of the future vessel, SM22α-lacZ expression was confined to the ventral and lateral walls and most posterior only ventral walls. The HoxB6-Cre/R26 reporter was expressed in splanchnic and somatic lateral plate mesoderm. All cells in the ventral part of the aorta (including SM22α-lacZ-positive cells) expressed the reporter at three investigated levels between the forelimb and hindlimb regions. The reporter was also expressed in a single layer of cells on the lateral and dorsal sides of the vessel. By transmission electron microscopy (TEM) the identity of these cells could be determined. They were shown to be endothelial cells. No peri-endothelial cells on the lateral or dorsal sides of the vessel expressed the HoxB6-Cre/R26 reporter. On the ventral side of the vessel on the other hand, it was shown that both endothelial cells and peri-endothelial cells expressed the reporter.

In order to follow the fate of the lateral plate mesoderm-derived cells in the descending aorta, E11.5 HoxB6-Cre/R26 embryos were studied. By this time, the lateral plate mesoderm-derived cells that occupied the ventral part of the vessel wall at E9.5 had been replaced by cells of other origin. A single layer of lacZ-positive cells was detected around the vessel at E11.5 whereas surrounding tissues were unstained. In order to determine the identity of the cells within this lacZ-positive layer of cells, triple staining for SM α-actin, PECAM-1 (CD31) and the R26 reporter (lacZ) was performed. It was shown that SMCs were not lacZ-stained, while endothelial cells in general were.

SMCs in the adult descending aorta are not of lateral plate mesoderm origin.

In adult HoxB6-Cre/R26 reporter mice the endothelial cell layer expressed the reporter. SMCs in the anterior and middle part of the vessel were unstained. In the part of the vessel posterior to the renal arteries however, endothelial cells as well as SMCs expressed the reporter. The interpretation of this finding is unclear. Either, lateral plate mesoderm actually contributes to the population of SMCs in the posterior part of the vessel, or the finding may be a result of ectopic expression of the reporter in this part of the body. The expression pattern seen in the posterior part of the body could correspond to an ectopic expression in paraxial mesoderm at E9.0.

At E10.5 lateral plate mesoderm-derived SMCs in the ventral wall are replaced by somite-derived cells.

In order to determine the identity of the cells that replaced the lateral plate mesoderm-derived SMCs at E11.5, Meox1-Cre/R26 reporter mice were studied. With these mice it was possible to detect somite contribution to the aorta. The Meox1 gene is expressed in pre-somitic and paraxial mesoderm and in the somites (Candia, Hu et al. 1992). It activates the R26 reporter in somites and somite-derived tissues from E8.5-E9.5 in Meox1-Cre/R26 mice (Jukkola, Trokovic et al. 2005). At E9.5, reporter expression could be detected in myodermatome and sclerotome and in the dorsal and dorsolateral walls of the aorta. No expression was seen in the ventral or ventrolateral walls. At E10.5 the reporter gene was still homogenously expressed in dorsal mesoderm. At this point however reporter-expressing cells had migrated along the ventrolateral walls of the aorta to populate also ventral positions. At E10.5, the aorta was entirely circumvented by somite-derived cells; cells of other origin had been replaced. This finding corresponds to the expression pattern in the HoxB6-Cre/R26 mice; the lateral plate mesoderm-derived SMCs that disappeared between E9.5 and E11.5 were replaced by cells of somitic origin. Furthermore, in the posterior part of E10.5 embryos, cells in the ventral aspect of the vessel wall were found not to express the reporter. This expression pattern suggests that the dorsal-to-ventral migration and replacement of cells is initiated in the anterior part of the body and progresses posteriorly.

In the adult mouse aorta, SMCs are somite-derived.

Aortas from postnatal and adult Meox1-Cre/R26 mice were dissected, whole mount X-gal stained and examined. It was found that all SMCs in adult aortas - from cardiac outflow tract to iliac arteries – expressed the reporter. It was furthermore discovered in postnatal pups that coeliac and superior mesenteric arteries were not derivatives of the somites, while renal and intercostals arteries were. The border between stained and unstained structures was distinct at the branch points examined.

Ectopic expression of Meox1-Cre/R26 was detected in the cardiac outflow tract and in the kidneys.

In order to further determine possible limitations of the chosen approach of cell lineage tracing, the expression pattern of the endogenous Meox1 gene was studied in more detail. The temporal expression of Meox1 mRNA was studied in E9.5-E18.5 whole embryo extracts by quantitative RT-PCR. It was found that the expression of Meox1 mRNA peaked at E9.5-E10.5 during somite formation. Thereafter expression declined rapidly although it was sustained at measureable levels to the end of embryogenesis. In whole mount X-gal stainings of organs from Meox1-Cre/R26 P2 pups, expression was restricted to somite-derived tissues like skeletal muscle, bone, cartilage, dermis and endothelial cells. Some ectopic expression was seen in cardiac outflow tract, in the kidney cortex and in some cells building a fine branched network on the heart surface, however not coronary arteries. Candia et al. described a Meox1 expression in branchial arch mesenchyme that was compatible with ectopic expression of the reporter in cardiac outflow tract (Candia, Hu et al. 1992). The developmental origin of the cells in cardiac outflow tract has been thoroughly investigated by others (Jiang, Rowitch et al. 2000). It is mainly derived from neural crest. Neural crest also gives rise to the cardiac conduction system which have an appearance very similar to the branched network we saw on the heart surface (Jiang, Rowitch et al. 2000). The kidney, adrenals and associated structures have traditionally been regarded as derivatives from intermediate mesoderm. The staining pattern described in our study indicates an ectopic expression of the reporter in a part of the intermediate mesoderm called the metanephrogenic blastema (Sariola and Sainio 1998).

3.4.2. Paper II

LPP has been identified as a SMC marker (Nelander, Mostad et al. 2003), (Gorenne, Nakamoto et al. 2003). It has been shown to regulate SMC migration (Gorenne, Jin et al. 2006) as well as to take part in transcriptional regulation (Guo, Sallis et al. 2006). It has furthermore been suggested that LPP would play an important role in atherosclerosis (Gorenne, Jin et al. 2006). In order to learn more about vascular SMCs and the proposed role of LPP in physiological and pathological conditions, the transcriptional regulation of LPP was studied. As mentioned before, the protein SRF acts by binding to sequences called CArG boxes in the genome. CArG boxes can be found in several SMC marker gene promoter regions. Since transcriptional regulation by SRF is common for SMC markers (Miano 2003), we investigated whether the transcription of the LPP gene would be directly regulated by CArG boxes and SRF.

The LPP gene contains three evolutionarily conserved CArG boxes, of which CArG 8 is associated to the promoter region of a previously unknown alternative promoter.
Bioinformatic studies showed that the mouse LPP gene (including 100 kb of flanking sequence) contained in total 35 putative CArG boxes. After evaluation, three of these CArG boxes were found to be evolutionarily conserved: CArG 8, CArG 11 and CArG 13. Previously identified CArG boxes have been located close to the promoter and within 4 kb of transcription start sites (TSSs). The conserved CArGs that we found in LPP were however situated far downstream from (by then) the only known TSS in LPP. The LPP gene was screened for alternative TSSs, using certain databases and software screening tools. The presence of a possible alternative promoter region close to the predicted CArG 8, together with an alternative first exon (named 2b) within intron 2, were hereby discovered. RT-PCR could confirm the presence of alternative exon 2b-containing transcripts in smooth muscle-rich tissues. It could also be concluded after careful primer design that the alternative transcript was not a splice variant, but a product of an alternative promoter.

The alternative promoter is active in smooth muscle-rich tissues.

By TaqMan RT-PCR the tissue-specificity of the alternative and upstream promoters could be compared. Primers were designed to target either the border between exons 2a-3 or exons 2b-3. Expressions of SM-MHC and total LPP were used as comparisons. It was found that the alternative exon 2b-containing transcripts were present in smooth muscle-rich tissues like bladder, aorta, stomach and gut, while the exon 2a-containing transcripts appeared without such preference. The function of the alternative promoter 2b seems to be to specifically direct LPP expression to SMCs. Since LPP is known as a SMC marker, the alternative promoter furthermore appears to be the primary regulator of LPP transcription in general.

SRF is able to bind to the sequences of LPP CArG 8, CArG 11 and CArG 13, but in the context of intact chromatin, it will only interact with CArG 8 and CArG 13.

Electrophoretic mobility shift assays (EMSA) were used to determine whether SRF could bind to the three evolutionarily conserved LPP CArG boxes we had found. It was confirmed that SRF in nuclear extracts bound to radiolabeled CArG sequences. On an electrophoretic gel, the hereby formed protein-DNA complexes were supershifted in the presence of an SRF antibody. A chromatin immunoprecipitation (ChIP) was also performed to determine if SRF could bind to the same endogenous LPP CArG boxes also in genomic DNA. Chromatin from cultured mouse aortic SMCs was cross-linked and divided into small pieces by sonication. It was then precipitated with an SRF antibody and the bound DNA fragments were further analyzed by PCR. It was concluded that SRF was able to bind CArG 8 and CArG 13 in cultured mouse aortic SMCs. Interestingly, none of the CArG boxes (CArGs 1-5) situated in the vicinity of the previously known upstream promoter, were precipitated by SRF in this experiment.

The alternative promoter activates transcription in aortic SMCs. CArG 8 promotes transcriptional activity when exposed to SRF/myocardin.

In order to determine the putative promoter activity of the conserved CArG boxes CArG 8, CArG 11 and CArG 13, constructs including these CArG sequences were cloned into a vector (pGL3basic) and tested in a luciferase reporter assay. It was

shown that the CArG 8-containing construct increased luciferase activity 40-fold, compared with empty vector. The experiment was performed after transfection into aortic SMCs. When CArG 8 was deleted from the construct, a dramatic decrease of luciferase activity was seen. CArG 11 and CArG 13 showed low or no promoter activity in this assay. The possible role of CArG 8, CArG 11 and CArG 13 as *enhancers* of transcription was then tested by subcloning them into a vector that contained a functional promoter (pGL3promoter). CArG 13 as enhancer showed a 4-fold increase of luciferase activity – an effect assignable to the CArG sequence. CArG 8 and CArG 11 were not shown to work as enhancers in this assay. To further investigate the effect of SRF/myocardin, the responsiveness of CArG 8-containing fragments to SRF and myocardin was tested, respectively. It was found that when levels of SRF were increased in the nucleus by overexpression, CArG 8-dependent transcription in this assay was downregulated. Downregulation of transcription did not appear if the CArG 8 sequence had been deleted. Since SRF is known as a transcriptional *activator*, the result was surprising. The phenomenon could be explained by so called squelching (Prywes and Zhu 1992), i.e. when a common coactivator used by multiple transcriptional activators is bound to and "consumed" by the excess of SRF. Overexpression of myocardin induced the promoter activity 4-fold in this assay, an effect that completely disappeared if the CArG 8 sequence was deleted. It was concluded that the alternative promoter acted as an activator of transcription in aortic SMCs. CArG 8 was furthermore important for the promoter response seen in these cells when levels of SRF/myocardin in the nucleus were increased.

Absence of endogenous SRF downregulates the exon 2b transcript levels. The alternative promoter is the main target for SRF-dependent LPP transcription in SMCs under physiological conditions.

SRF-deficient ES cells (ES100) (Schratt, Philippar et al. 2002) and smooth muscle-specific SRF knockout mice (Angstenberger, Wegener et al. 2007) were used to investigate whether the absence of endogenous SRF would affect exon 2b transcription under physiological conditions. In $SRF^{-/-}$ ES cells exon 2b expression was reduced 70%, compared with WT ES cells. This reduction could be compensated by overexpression of the constitutively active SRF fusion protein SRF-VP16. As a

comparison, the level of exon *2a* transcripts was similar in $SRF^{-/-}$ ES cells and WT ES cells. Overexpression of SRF fusion protein had only modest compensating effect on exon *2a* transcription. These data suggest that changes in SRF levels will affect 2b transcription rather than *2a* transcription. SRF-deficient mice tissues were then analyzed. At first, it was confirmed that the conditional smooth muscle-specific SRF knockout used had worked; SRF was downregulated in colon, bladder and colonic SMCs. As a control, SRF levels in heart were not affected by the genetic manipulation. The SRF-deficient mice tissues and cells were all found to have substantial decreases in exon 2b transcript levels: colon 90%, bladder 85% and colonic SMCs 75%. Although exon 2a levels also were affected to a lesser extent in this study, taken together, our results demonstrate that LPP expression in SMCs is directed by SRF/myocardin, through a previously unknown promoter generating exon 2b transcripts.

3.4.3. Paper III

Atherosclerosis is one of the leading causes of death in the Western world. Migration and proliferation of vascular SMCs are important pathophysiological steps in the development of atherosclerosis (Ross 1999). PDGF and Ang II have both been linked to the initiation and progression of atherosclerosis. PDGF stimulates proliferation (Ross, Glomset et al. 1974) and migration (Grotendorst, Chang et al. 1982), (Seppa, Grotendorst et al. 1982) of SMCs in vitro. Ang II plays an important role in cardiovascular and renal homeostasis. Besides its physiological effects on renal sodium reabsorption and its action as vasoconstrictor, Ang II induces cellular hypertrophy in cultured vascular SMCs (Geisterfer, Peach et al. 1988), (Berk, Vekshtein et al. 1989). Cultured vascular SMCs have also been shown to migrate towards an Ang II gradient; Ang II increases vascular SMC migration compared with unstimulated cells (Xi, Graf et al. 1999). Ang II infusion to $apoE^{-/-}$ mice has furthermore been shown to result in an increased severity of aortic atherosclerotic lesions (Daugherty, Manning et al. 2000). It has been suggested that Ang II stimulation on SMC migration would be mediated by PDGF-B and that Ang II may act by stimulating the PDGFR-β (Kim, Zhan et al. 2000), (Nadal, Scicli et al. 2002), (Eskildsen-Helmond and Mulvany 2003). In agreement with that, it was shown in rat

that administration of an Ang II receptor antagonist reduced mRNA levels of PDGF-A and PDGF-B in balloon-injured arteries (Abe, Deguchi et al. 1997).

Haemodynamic factors like reduced shear stress have been suggested to be of importance for the progression of intimal hyperplasia and atherosclerosis. Within the vascular tree, certain predilection sites for intimal pathology have been identified. At vessel branch points and in the carotid bulb for example, atherosclerosis develops prematurely. At these sites the endothelium is physiologically exposed to low wall shear stress or flow separation (Friedman, Hutchins et al. 1981), (Zarins, Giddens et al. 1983). Atherosclerosis-like conditions and neointima formation can be created artificially in animal models by damaging the endothelium or reducing blood flow by surgical manipulation. In a study performed in rat (Mondy, Lindner et al. 1997), proliferation and PDGF expression were studied in endothelial cells after reduced carotid blood flow. It was found that endothelial cells proliferated more and had a higher expression of PDGF-B in the reduced blood flow vessel. In a balloon denudation study in rat, it was shown that the expression of PDGF-B was increased in injured vessels and that inhibition of PDGF receptor β (PDGFR-β) expression by an antisense oligonucleotide, reduced neointima formation (Sirois, Simons et al. 1997). In a similar study, performed in rabbit, administration of PDGF-receptor inhibitor imatinib caused a reduction of neointima formation due to increased intimal SMC apoptosis. However, this effect was of short duration and lesions reoccurred (Leppanen, Rutanen et al. 2004).

In the study presented here, our aim was to investigate the possible roles of PDGF-B and Ang II in an atherosclerosis-like process in vivo. A mouse model of carotid artery ligation was used (Kumar and Lindner 1997). Low blood flow was hereby created and a neointima was established. After ligation, animals were exposed to different combinations of Ang II infusion and/or treatment with PDGFR-β inhibitor (imatinib). The following study design was used: i) untreated control, ii) PDGFR-β inhibition iii) Ang II infusion, iv) PDGFR-β inhibition *and* Ang II infusion. On day 14 from ligation, all animals were sacrificed. Carotid arteries were excised and analyzed histologically. Vessels from groups i) and iii) were prepared to measure PDGF-B and PDGFR-β mRNA expression levels with real-time PCR. It was shown that PDGF-B

was upregulated in ligated compared with unligated vessels. Ang II infusion had no further effect on PDGF-B mRNA expression. The expression of PDGFR-β mRNA was not affected by artery ligation or by Ang II infusion. The increase of intimal area seen in ligated vessels was not affected by administration of imatinib, indicating a neointima formation independent of PDGFR-β signaling. Infusion of Ang II however resulted in an accelerated intimal hyperplasia compared with ligated control vessels. These experiments were performed in 10 weeks old male C57Bl6 mice. To further analyze the importance of PDGF in neointima formation, ligations were performed in homozygous PDGF-$B^{ret/ret}$ retention motif knockout mice (RetKO) (Lindblom, Gerhardt et al. 2003) in parallel (constituting group v) with littermate control group vi)). RetKO mice lack the PDGF-B retention motif, a C-terminal amino acid sequence at the end of the PDGF-B protein. This condition will prevent synthesized PDGF-B proteins from being accumulated on the cell surface or in the near extracellular matrix, bound to heparin sulphate proteoglycans. Instead, PDGF-B proteins in RetKO will be diffusible. Receptor-binding ability or biological activity of the protein is not affected in the recombinant protein (Ostman, Backstrom et al. 1989). Ligated vessels from RetKO mice displayed an increase of intimal area compared with unligated vessels. The intimal thickening seemed to be independent of the RetKO and appeared in littermate controls as well. In order to study SMC content in the neointima formed, vessels from RetKO mice v) and their control mice vi) were stained using SM α-actin. Ligated vessels were shown to have a high content of SMCs. There was however no difference in SMC content between RetKO and control mice.

In summary, we conclude that ligation of carotid arteries induced local neointima formation. Infusion of Ang II accelerated the process. In the intimal thickenings seen, PDGF-B expression was elevated compared with a normal vessel. The expression of PDGFR-β was unaffected in ligated vessels and neither inhibition of PDGFR-β or knockout of the retention motif had any effect on neointima formation.

3.5. Conclusions

Cardiovascular diseases like atherosclerosis are serious conditions causing suffering to the individual and high costs to society. One proposed key player in the initiation and progression of atherosclerosis is the vascular SMC. In this thesis, effort was put on investigating the developmental origin of vascular SMCs and to learn more about SMC transcriptional regulation. The effect of PDGF-B and Ang II on SMCs in an atherosclerosis-like situation was also studied.

The origin of vascular SMCs in the descending mouse aorta was determined by means of transgenic mice and cell lineage tracing. Aortic SMCs were found to be solely somite-derived. This finding is in contrast to what has previously been the dogma. Until recently, it has generally been assumed that vascular SMCs would be of lateral plate mesoderm origin, although experimental evidence for such theory has been lacking (Gittenberger-de Groot, DeRuiter et al. 1999). Furthermore, a ventral-to-dorsal migration of vascular SMCs during development has been assumed. It has been suggested that mesenchymal cells in the ventral part of the future aorta are induced to SMC differentiation and later migrate to populate dorsal positions. The migration hypothesis was based on the fact that the earliest signs of SMC differentiation are seen in the ventral part of the vessel wall (Hungerford, Owens et al. 1996), (Takahashi, Imanaka et al. 1996). Our data confirm that the first signs of SMC differentiation are seen (at E9.0 in mice) in the ventral part of the vessel wall and shortly after in dorsal and lateral positions. Moreover, the first signs of SMC differentiation appear in cells of lateral plate mesoderm origin. However, by our cell lineage tracing experiments it was determined that the suggested ventral-to-dorsal migration of cells does not appear. Moreover, the lateral plate mesoderm-derived SMCs in the ventral aspect of the aorta are replaced at E10.5 by cells of somitic origin. These somite-derived cells have performed a dorsal-to-ventral migration (Figure 2). Lineage tracing experiments with transgenic Cre mice have limitations however. It is impossible to guarantee that the Cre strain chosen expresses the reporter in a strict lineage pattern. In the experiments presented here, ectopic expression might take place in non-somite-derived tissues, which theoretically could contribute to the vascular SMC population. This contribution would be a non-lineage contribution, hard to trace. Such arguments weaken the Meox1-Cre/R26R results. Our results are

however supported by experiments in other species. In chicken-quail chimaeras, Pouget et al. could demonstrate a somite contribution to endothelial cells and vascular SMCs in the aorta (Pouget, Gautier et al. 2006). The somitic contribution has been further specified to be of sclerotomal origin (Wiegreffe, Christ et al. 2007), (Pouget, Pottin et al. 2008). The results from the HoxB6-Cre/R26R mice are less problematic since the conclusion drawn builds on the absence of cells. We claim that lateral plate mesoderm does not contribute to the aorta. Ectopic expression of the reporter is therefore not a problem. The lateral plate mesoderm origin of the aorta has been a

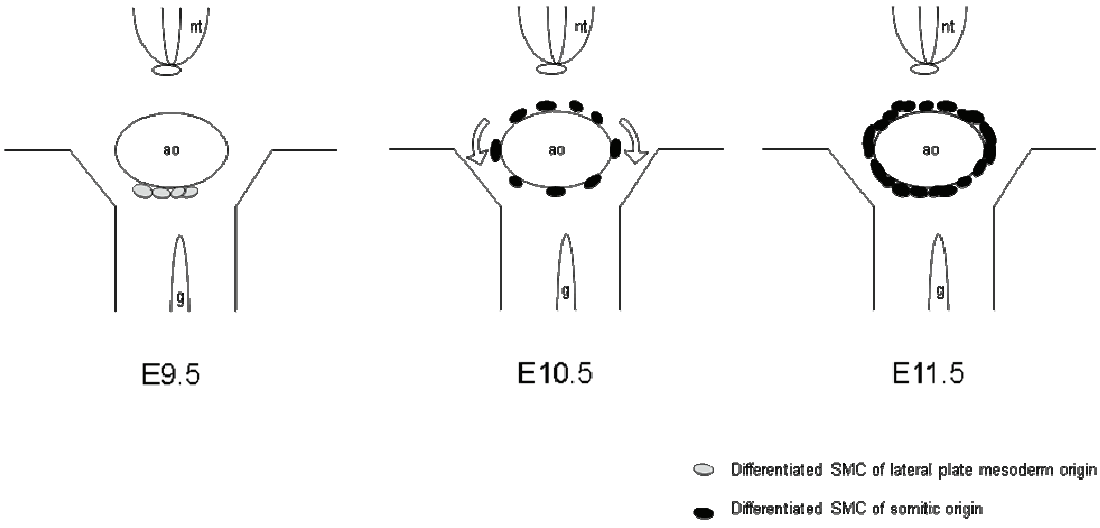

Figure 2. Aortic SMCs are derived from the somites. Somite-derived SMCs perform a dorsal-to-ventral migration whereby lateral plate mesoderm-derived SMCs on the ventral side of the aorta are replaced by somite-derived SMCs at E10.5. (nt=neural tube, ao=aorta, g=gut)

dogma for a long time. Our results clarify that lateral plate mesoderm, in contrast to the widely accepted hypothesis, does not contribute to vascular SMCs in the aorta. Our data from transgenic mice are in line with the recent results in chicken. It is now possible to map the developmental origins of aortic SMCs with greater precision. Such knowledge is valuable when one should try to understand SMC induction, interpret different knockout mice or evaluate SMC origin as a susceptibility factor for vascular disease. In 2000, the Edg1 knockout mouse was described by Liu et al.

Edg1-deficient mice display a lack of SMCs in the dorsal wall of the aorta. According to the authors the phenotype was attributable to a defect SMC migration from a ventral pool of progenitors (Liu, Wada et al. 2000). Our results however call for a new interpretation of the Edg1 knockout phenotype as well as for a complete revision of the migration theory.

The cell type "smooth muscle" was for long defined on morphological criteria alone. SMCs however constitute a heterogeneous population of cells with a plastic phenotype. The interest for SMC marker genes and the transcriptional regulation of these genes have therefore increased. In this thesis, the transcriptional regulation of SMC-specific protein lipoma preferred partner (LPP) was investigated. Lim-domain protein LPP has been shown to associate to the cytoskeleton at focal adhesions and dense bodies (Gorenne, Nakamoto et al. 2003), (Petit, Fradelizi et al. 2000), (Petit, Meulemans et al. 2003). It participates in the regulation of SMC migration (Gorenne, Jin et al. 2006) and shuttles to the nucleus (Petit, Fradelizi et al. 2000) where it has been shown to have a transactivating capacity (Guo, Sallis et al. 2006). In neointima formation after vascular injury, LPP is co-expressed with SM α-actin and it has been suggested that LPP might play a role in the development of atherosclerosis (Gorenne, Jin et al. 2006). In this thesis, it was shown that the LPP gene contains 3 evolutionary conserved cis-regulatory elements called CArG boxes. One of them was shown to be part of the LPP transcriptional machinery. Interestingly, none of the CArGs in the vicinity of the previously known upstream promoter were able to bind SRF in our experiments. It was shown that in smooth muscle-rich tissues, LPP transcription is directed by an alternative, previously unknown promoter, within intron 2. The alternative promoter produces an alternative exon 2b transcript. Our results supplement what has previously been known about LPP transcription. In general, CArG boxes are located within 4 kb from transcription start sites. For LPP it has been assumed that LPP transcription starts at exon 1 and that only one transcript is produced. We have shown that the LPP gene is able to generate two transcripts, i.e. two different sequences that are not splice variants of a single transcript. The two transcripts furthermore appear to be differentially expressed. Our results do not offer any formal evidence that the alternative promoter is required for SMC-specific expression of LPP. The promoter described in our work however appears to be

expressed exclusively in SMCs: bladder, aorta, stomach and gut. Contrary, the transcript from the upstream promoter is expressed throughout the body. In this thesis it was furthermore demonstrated that LPP transcription is SRF-dependent. We found a downregulation of LPP exon 2b transcripts in SRF-deficient ES cells and smooth muscle tissues. The importance of SRF has been shown for several other SMC marker genes (Miano 2003). In general, SRF participates in transcriptional programs for growth and muscle differentiation. It acts by binding to CArG boxes. Studies of SRF-deficient smooth muscle tissue in mice (Miano, Ramanan et al. 2004) have shown that SRF is associated with proper assembly of cytoskeletal and contractile elements and terminal differentiation of muscle. SMCs in the dorsal aorta lacked expression of smooth muscle marker SM-MHC in these mice, indicating a less differentiated state of smooth muscle when SRF was absent. Miano et al. also found a reduced number of SMCs in the dorsal aspect of the aorta and suggested it should be explained by a defect cell migration. It has previously been shown that SRF-deficient ES cells have impaired migration (Schratt, Philippar et al. 2002). Our results incorporate LPP into the large group of SRF-dependent smooth muscle marker genes. Some of the effects seen in SRF-deficient tissues may be attributable to properties of LPP. The next step should be a detailed description of the in vivo functions of LPP, including the significance of having two different transcripts produced by the LPP gene.

In this thesis, a carotid artery ligation model was used to evaluate the proposed roles of PDGF-B and Ang II in neointima formation. It was reported that administration of Ang II to ligated vessels increased neointima formation further and that it was an effect independent of PDGFR-signaling. Ang II infusion to atherosclerosis-prone apoE$^{-/-}$ animals have previously been shown to create more severe lesions (Daugherty, Manning et al. 2000). The stimulating effect by Ang II reported in this thesis is in line with the effect on cultured vascular SMCs; Ang II induces cellular hypertrophy in cultured vascular SMCs (Geisterfer, Peach et al. 1988), (Berk, Vekshtein et al. 1989). It has been suggested by others that the Ang II effect would be mediated by PDGFR-β (Kim, Zhan et al. 2000), (Nadal, Scicli et al. 2002), (Eskildsen-Helmond and Mulvany 2003). The influence Ang II had on neointima formation was however unaffected by PDGF-receptor inhibition. It could be argued that no experiment was performed to ensure that the inhibition had been successful. In vitro studies have shown that PDGF

stimulates proliferation and migration (Ross, Glomset et al. 1974) (Grotendorst, Chang et al. 1982), (Seppa, Grotendorst et al. 1982). In this thesis it was shown that PDGF-B mRNA expression increased in ligated vessels compared with unligated. The role of PDGF-B signaling in neointima formation is however unclear since inhibition of the PDGFR-β as well as knockout of the PDGF-B retention motif had no effect.

4. Acknowledgements

I would like to thank everyone who has contributed to this thesis. In particular I would like to thank my supervisor Per Lindahl. You have always been willing to discuss all different aspects of biology and your great enthusiasm for science has been inspiring. Our co-authors are also acknowledged for valuable contributions. I moreover thank former and present members of the Lindahl group for your experimental and social support. In particular I would like to acknowledge my lab-room-mate Henrik Lindskog for your encouraging remarks and good temper. I am furthermore grateful to all friends and colleagues at the Wallenberg Laboratory and at the Department of Medical Biochemistry and Cell Biology who have showed interest in my projects and supported me during this period. Finally, I would also like to thank Christer Betsholtz, Ove Lundgren and Anders Vahlquist. You accepted me as an undergraduate "summer-researcher" at your labs, which gave me useful introductions to experimental work in general and exciting insights into your specific fields of research.

This thesis was supported by The Göteborg Medical Society and Sahlgrenska University Hospital.

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