Regulation of gene expression in the vascular wall

Erik Larsson

UNIVERSITY OF GOTHENBURG

Department of Medical Biochemistry and Cell Biology
Institute of Biomedicine
Wallenberg Laboratory for Cardiovascular Research
Sahlgrenska Academy

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Abstract

Blood vessel growth and function are closely related to a number of pathological conditions, including tumor angiogenesis, wound healing and atherosclerosis. Smooth muscle cells (SMC) and endothelial cells (EC), the two major constituents of the vascular wall, are both characterized by the expression of unique phenotypic marker genes, many of which have vital roles in blood vessel development and disease. We therefore sought to obtain a more complete picture of vascular-specific gene expression, gene regulation and genetic variation.

We performed an unbiased computational screen to identify cases of transcriptional coregulation in mammalian cell differentiation. This generated a number of novel hypotheses, one of them being that the SMC marker gene lipoma-preferred partner (LPP) could be activated by serum response factor (SRF), a known master regulator of SMC differentiation. Using chromatin immunoprecipitation, gel shift assays, reporter assays and transgenic mouse models, we showed that LPP belongs to the category of SMC-specific genes that are regulated by SRF, an important insight because LPP has a role in the control of SMC migration.

By combining in-house and public genome-wide expression data, we identified 32 novel EC-specific mRNAs. A number of these, such as the G-protein coupled receptors Gpr116 and Ramp2, represent putative drug targets. By integrating our results with data from published genome-wide association studies, we investigated if genetic variation in EC-specific genes contributes to human disease. Independent replication of selected SNPs in 10,505 individuals revealed that a variant in one of the novel EC markers, DRAM, is associated with the development of essential hypertension.

Finally, the role of microRNAs (miRNA), an abundant class of small regulatory RNAs, was evaluated in the microvasculature. Through screening of public expression data, we identified a novel microvascular-enriched miRNA, miR-145, and showed that overexpression of this molecule leads to reduced cell migration.

In conclusion, we identified novel vascular marker genes and provided insights into the regulation of such genes. In addition, we showed that genetic variation in a novel EC marker gene contributes to the development of hypertension in the human population.

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Predictive screening for regulators of conserved functional gene modules (gene batteries) in mammals
Nelander S, Larsson E, Kristiansson E, Mansson R, Nerman O, Sigvardsson M, Mostad P, Lindahl P
BMC Genomics, 2005. 6(1):68

II. SRF regulates transcription of the smooth muscle marker LPP via an alternative promoter
Petit M*, Lindskog H*, Larsson E*, Wasteson P, Nelander S, Athley E and Lindahl P
Circ Res, 2008. 103(1):61-9
*These authors contributed equally

III. Identification of a core set of 58 gene transcripts with broad and specific expression in the microvasculature
Wallgard E, Larsson E, He L, Hellström M, Armulik A, Nisancioglu MH, Genove G, Lindahl P and Betsholtz C
Arterioscler Thromb Vasc Biol, 2008. 28(8):1469-76

IV. The DRAM locus at 12q23 is associated with hypertension
Larsson E, Wahlstrand B, Hedblad B, Hedner T, Kjeldsen S, Melander O and Lindahl P
Submitted

V. Screening for microvascular miRNAs: miR-145 is enriched in microvessels, is expressed in pericytes and is a regulator of Fli1
Larsson E, Fredlund-Fuchs P, Bondjers C, Barkefors I, Genove G, Heldin J, Harvey SJ, Kreuger J and Lindahl P
Manuscript

Abbreviations

ASMA	Smooth muscle α-actin
BLAST	Basic local alignment and search tool
BrdU	Bromodeoxyuridine
ChIP	Chromatin immunoprecipitation
DLL4	Delta-like ligand 4
DNA	Deoxyribonucleic acid
DRAM	Damage-regulated autophagy modulator
dsRNA	Double-stranded RNA
EC	Endothelial cell
ECM	Extracellular matrix
EMSA	Electrophoretic mobility shift assay
FDR	False discovery rate
FLK1	Fetal liver kinase 1 (VEGFR2)
FLT1	FMS-like tyrosine kinase 1 (VEGFR1)
FOX	Forkhead box factor
GPCR	G-protein coupled receptor
GWA	Genome-wide association
HIF1α	Hypoxia-inducible factor 1α
HT	Hypertension
HUVEC	Human umbilical vein endothelial cells
KDR	Kinase insert domain receptor (VEGFR2)
LD	Linkage disequilibrium
LPP	Lipoma-preferred partner
MDC-CC	Malmö diet and cancer study - cardiovascular cohort
miRNA	microRNA
mRNA	Messenger RNA
MRTFA	Myocardin-related transcription factor A
MRTFB	Myocardin-related transcription factor B
NORDIL	Nordic diltiazem study
NRP1	Neuropilin 1
NRP2	Neuropilin 2
PAR	Population attributable risk
PC	Pericyte
PCR	Polymerase chain reaction
PDGFB	Platelet-derived growth factor B
PDGFRβ	PDGF receptor β
PFM	Position frequency matrix
qRT-PCR	Quantitative RT-PCR
RNA	Ribonucleic acid

RT-PCR	Reverse transcription PCR
SM-MHC	Smooth muscle myosin heavy chain
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SRF	Serum response factor
TCE	TGF-β control element
TGF-β1	Transforming growth factor-β1
TSS	Transcription start site
UTR	Untranslated region
VAEC	Vascular aortic endothelial cells
VEGFA	Vascular endothelial growth factor A
VEGFR1	VEGF receptor 1
VEGFR2	VEGF receptor 2
VSMC	Vascular SMC
WTCCC	Welcome trust case-control consortium

Table of contents

1 Introduction

1.1 Brief overview of this thesis

This thesis deals with gene expression and gene regulation in the blood vessel wall. For the convenience of the reader, a brief introduction to gene regulation, with special emphasis on vertebrates, is given in Section 1.2. It continues with a general introduction to blood vessel development and the basic molecular mechanisms that are involved in this process (Sections 1.3.1-1.3.4). In addition, previous work on the transcriptional regulation of differentiation marker genes of smooth muscle cells (SMC) (Section 1.3.5) and endothelial cells (EC) (Section 1.3.6) is briefly summarized.

The present work is based on five papers, of which three are in published form and two are presented as manuscripts. These are included as an appendix and are referred to by their Roman numerals (**Paper I-V**) in the text. The major findings of each paper are summarized and discussed in Section 3. Concerted activation of functionally coupled genes by shared regulatory programs is a central mechanism in mammalian cell differentiation. **Paper I** describes how common regulators of coexpressed genes can be discovered using computational methods, and several new hypotheses are proposed therein. One of these hypotheses, the possibility that the SMC-specific regulator of cell migration lipoma-preferred partner (LPP) is regulated by serum response factor (SRF), is investigated in **Paper II**. The results show that LPP is a novel SRF target, and that SMC transcription of LPP is mediated by SRF binding to an alternative, intronic, promoter in LPP. **Paper III** focuses on specific gene expression in the endothelium. Through meta-analysis of public and in-house expression datasets, more than 20 novel EC differentiation markers are identified. Several of these genes encode possible drug targets such as G-protein coupled receptors (GPCR), and the results may therefore be relevant in the development of future angiogenesis-related therapies. Recent developments in high-throughput genotyping have enabled genome-wide association studies, where hundreds of thousands of genetic variations are screened for disease association. In **Paper IV**, we hypothesize that genetic variation in genes expressed specifically in the endothelium may be important in the development of common diseases. Data from a recently published GWA study was therefore combined with results from Paper III, and this resulted in the discovery of a novel hypertension-associated single nucleotide polymorphism (SNP) in the *DRAM* (damage-related autophagy modulator) locus. MicroRNAs (miRNA) have recently emerged as key players in the regulation of gene expression. In **Paper V**, novel microvascular-enriched miRNAs are identified through mining of public expression data (using similar principles as in Paper III). Among other things, the study shows that miR-145 is a

marker for pericytes in microvessels and has a potential role in regulation of cell migration.

Finally, in Section 4, some final notes and future perspectives are given, and this is followed by an Acknowledgments section (Section 5) in which a large number of people who contributed to this work in various ways are given credit.

1.2 Regulation of gene expression in higher eukaryotes

1.2.1 Regulation of RNA polymerase II transcription

The human genome contains ~21.000 protein-coding genes – only a thousand more than the simple nematode worm, *C. elegans*. (ENSEMBL rel. 52, Dec. 2008) [1]. One can therefore argue that morphological and behavioral complexity does not primarily arise from an increased number of genes, but rather from the way genes are regulated [2]. Compared to *C. elegans*, mammalian genomes contain three times as many transcription factors ("*trans*-regulators"); proteins that bind DNA to regulate gene transcription in a sequence-dependent manner. In addition, the organization of regulatory DNA elements ("*cis*-regulators") is vastly more complex in vertebrates compared to lower organisms. Non-coding regulatory RNAs, often located in the 98% of the mammalian genome that does not code for proteins, add another layer of complexity to the gene regulatory network [3-5].

A mammalian gene usually contains several structured regulatory DNA regions ("enhancers"), each typically being ~500 bp long and containing binding sites for multiple transcriptional activators and repressors [2, 6]. These regions may be located tens of thousands of base pairs away from the transcription start site (TSS), in both upstream and downstream positions. Insulator regions, located between enhancers and promoters, can neutralize regulatory crosstalk between neighboring genes. Through intermediate regulatory complexes, enhancers modulate the assembly and activity of the basal transcription machinery on the core promoter. The core promoter, a 60-70 bp region flanking the TSS, contains one or several sequence elements (TATA, INR or DPE) required for recruitment of the so called general transcription factors – proteins such as TFIID that are required for binding and initiation of transcription by RNA polymerase II [7]. The elaborate organization of regulatory DNA in animals enables generation of complex expression patterns, but is also a complicating factor when performing regulatory sequence analysis.

1.2.2 Modular regulation, "gene batteries"

Terminal differentiation states, or cell types, can be defined in terms of differential expression of specific effector genes [8]. A commonly used concept is that of the "gene battery" – a set of functionally related genes that may be activated by similar *cis*- and *trans*-acting regulators [9]. The term dates back to geneticist Thomas H.

Morgan in the 1930's, i.e. before the determination of the structure of deoxyribonucleic acid (DNA) [10] and before the current molecular definition of a gene as a stretch of nucleic acid sequence that gives rise to a functional product [11]. Although it is clear that mammalian gene regulation is dauntingly complex and that the *cis*-regulatory logic of two coexpressed genes will never be identical, gene battery-like regulation has still been described as the dominant way that cell type-specific gene expression is achieved [8]. A considerable modularity in the organization of mammalian gene expression is immediately evident from the fact that transcription factors often are strongly associated with specific functional gene categories; consider e.g. coregulation of SMC marker genes by e.g. SRF and myocardin [12] (see Section 1.3.5), MyoD [13] and MEF2 [14] in the regulation of skeletal muscle genes and NRF1/2 and PGC1 in transcriptional control of respiratory chain components [15].

1.2.3 Post-transcriptional regulation by microRNAs

miRNAs are short endogenous RNAs that function as post-transcriptional regulators of gene expression in a range of biological processes [16]. Although the first described miRNA, lin-4 in *C. elegans*, was discovered already in 1993 [17], it is only during recent years that these molecules have emerged as a major class of regulatory RNAs [18]. Transcription of miRNA genes is carried out by RNA polymerase II, either as parts of introns in host protein coding genes or as dedicated miRNA genes [18]. Maturation begins with trimming of the immediate transcribed product (the pri-miRNA) into a 60-70 bp stem-loop structure (the pre-miRNA) by the nuclear enzyme Drosha [19]. Through a mechanism that depends on exportin-5, the pre-miRNA is exported from the nucleus and subsequently cleaved by the cytosolic enzyme Dicer into a short 19 to 25 bp double-stranded RNA. Normally, one strand is quickly degraded, while the other (the mature miRNA) associates with the RNA-induced silencing complex (RISC). This riboprotein complex has the ability to recognize and silence target mRNAs, usually through imperfect complementarity to sequence elements in the 3'-UTR [20]. miRNAs can induce both translational repression and destabilization/degradation of specific target mRNA transcripts. Since a single miRNA usually has hundreds or at least tens of target mRNAs, miRNAs are, in a sense, modular regulators. In addition, statistically significant functional coupling can in many cases be detected among targets of a specific miRNA [21, 22].

1.3 The blood vessel

1.3.1 Vasculogenesis and angiogenesis

Small, primitive organisms may rely on diffusion for the transport of substances within the living body. However, for larger distances this quickly becomes impractical [23], and in the complex body architecture of vertebrates, *blood vessels*

run through almost every organ and provide transport of oxygen, water, nutrients, signaling molecules and circulating cells. The vasculature is the first organ to form during development, and growth of the vascular system requires tight coordination of cell differentiation, proliferation, migration and signaling [24-26]. Early embryonic and yolk sac blood vessels form through differentiation of vascular progenitor cells of mesodermal origin, *angioblasts*, into endothelium (*vasculogenesis*) (Figure 1). Some vessels, like the dorsal aorta, develop directly from angioblasts, but in other places, such as the yolk sac, a primitive *vascular plexus* is formed. This is a uniform, immature network without vascular hierarchy. In order to form a functional vasculature, the plexus undergoes an extensive remodeling process, which involves proliferation, migration, pruning and sprouting in response to various molecular stimuli, thereby adapting blood supply to local requirements (*angiogenesis*). During this stage, endothelial tubes become covered with mural cells; *pericytes* appear around the smallest capillaries and *smooth muscle cells* (SMC) provide support and contractile properties to the larger vessels. Angiogenesis continues in the adult and enables normal tissue growth and repair, but also provides blood supply for cancerous tissue. A mature blood vessel has three major layers: an innermost endothelial monolayer lined by a basal lamina (intima), a supportive/contractile middle layer of SMCs and extracellular matrix (ECM), and an outer layer of loose ECM and fibroblasts. The capillary wall has a simpler architecture, consisting of endothelium, sparsely covered by supporting pericytes that are embedded in a basal lamina.

1.3.2 Molecular basis of blood vessel development

Vascular growth is controlled by a multitude of molecular signals, and knockout studies have revealed critical roles for a long list of genes, many of which are expressed specifically by endothelial cells (EC) or SMCs/pericytes [26] (Figure 2). In addition, a large number of knockout models show less severe vascular defects; at the time of writing of this thesis, the MGI mouse phenotype database reports 1309 genes with cardiovascular phenotypes of some sort [27]. Vascular endothelial growth factor A (VEGFA) is a central molecule in control of blood vessel growth and differentiation. VEGFA is part of a protein family that includes VEGFB, placental growth factor (PlGF), VEGFC and VEGFD [28]. In the early mouse embryo, vascular precursors appear in response to proteins such as bone morphogenetic protein 4 (BMP4) [29] as vascular endothelial growth factor receptor 2 (VEGFR2, KDR, FLK1) positive cells. These may give rise to both the hematopoietic and vascular lineages, and *Kdr* deficient mice die around embryonic day 9 due to failed development of blood vessels and hematopoietic cells [30]. Disruption of even a single allele of the major VEGFR2 ligand, VEGFA, also leads to embryonic death and a malformed vasculature [31, 32]. Vascular density needs to be adapted to local oxygen needs, and a key player here is the transcription factor

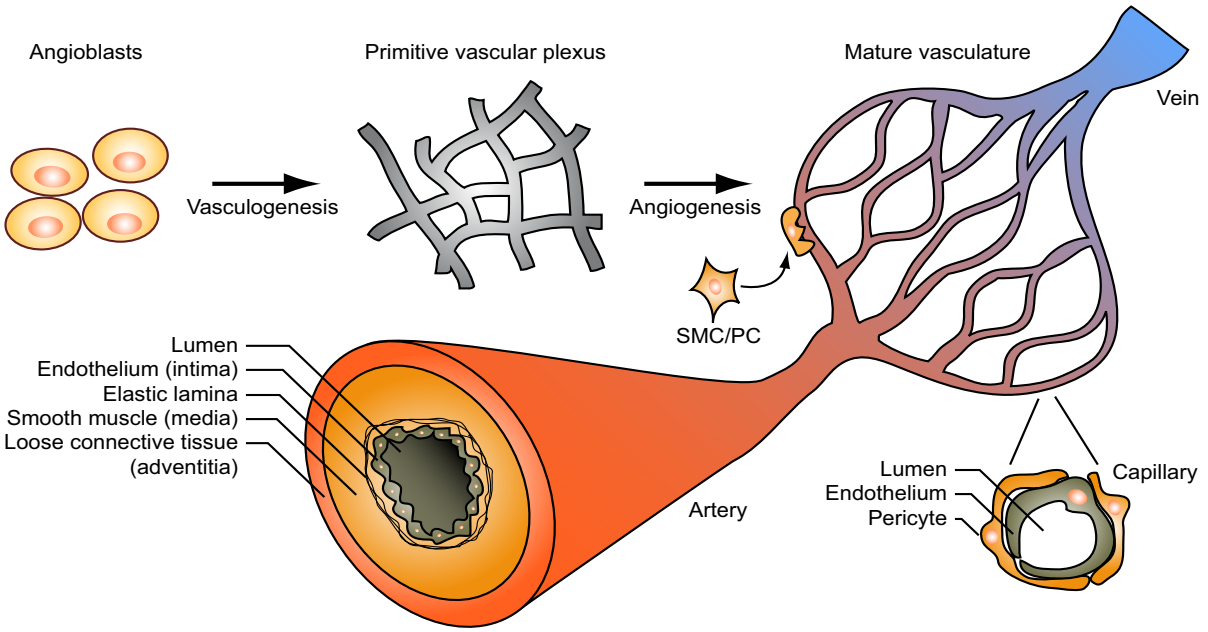


Figure 1. From vascular progenitor to mature vessel. Mesodermal vascular progenitors (angioblasts) differentiate and form a honeycomb-shaped vascular plexus in the embryo and in the yolk sac. After circulation is established, the plexus is adapted to local requirements. A typical mature artery has an inner monolayer of endothelial cells (the intima) that is lined by a basal lamina, followed by an elastin-containing elastic lamina. The middle layer (media) consists of multiple layers of smooth muscle embedded in matrix. The outermost layer (adventitia) consists of loose connective tissue and fibroblasts. The walls of capillaries consist of endothelial cells, sparsely surrounded by supporting pericytes. PC, pericyte; SMC, smooth muscle cell.

hypoxia-inducible factor 1α (HIF1α). In almost any cell type, hypoxic conditions lead to activation of HIF1α, which in turn triggers expression of hypoxic response genes, including VEGFA and other pro-angiogenic genes [33]. However, it seems clear that VEGFA expression during embryogenesis is also controlled by intricate hard-wired genetic programming; as an example, sonic hedgehog, a key developmental regulator, induces expression of VEGFA and other angiogenic factors [34]. In addition to VEGFR2, VEGFA can bind to a second receptor, VEGFR1 (FLT1), which does not have the potent angiogenic capabilities of VEGFR2. One function of this receptor may be to act as an inhibitory decoy, that reduces VEGFR2 activation by sequestering of VEGFA [35]. However, VEGFR1 also mediates specific downstream effects, such as induction of matrix metalloproteinases (MMPs) [36].

An early event during vascular maturation is the specification of veins and arteries. Arterial and venous endothelium appear to be molecularly distinct from each other already during the first stages of angiogenesis; expression of Ephrin-B2 marks arterial endothelium while its receptor Eph-B4 is an early marker for venous ECs [37]. The nuclear receptor COUP-TFII (*NR2F2*) is expressed in venous endothelium and seems to act further upstream in this process: in mice lacking the *Nr2f2* gene, vein ECs become arterial-like and express Ephrin-B2, while

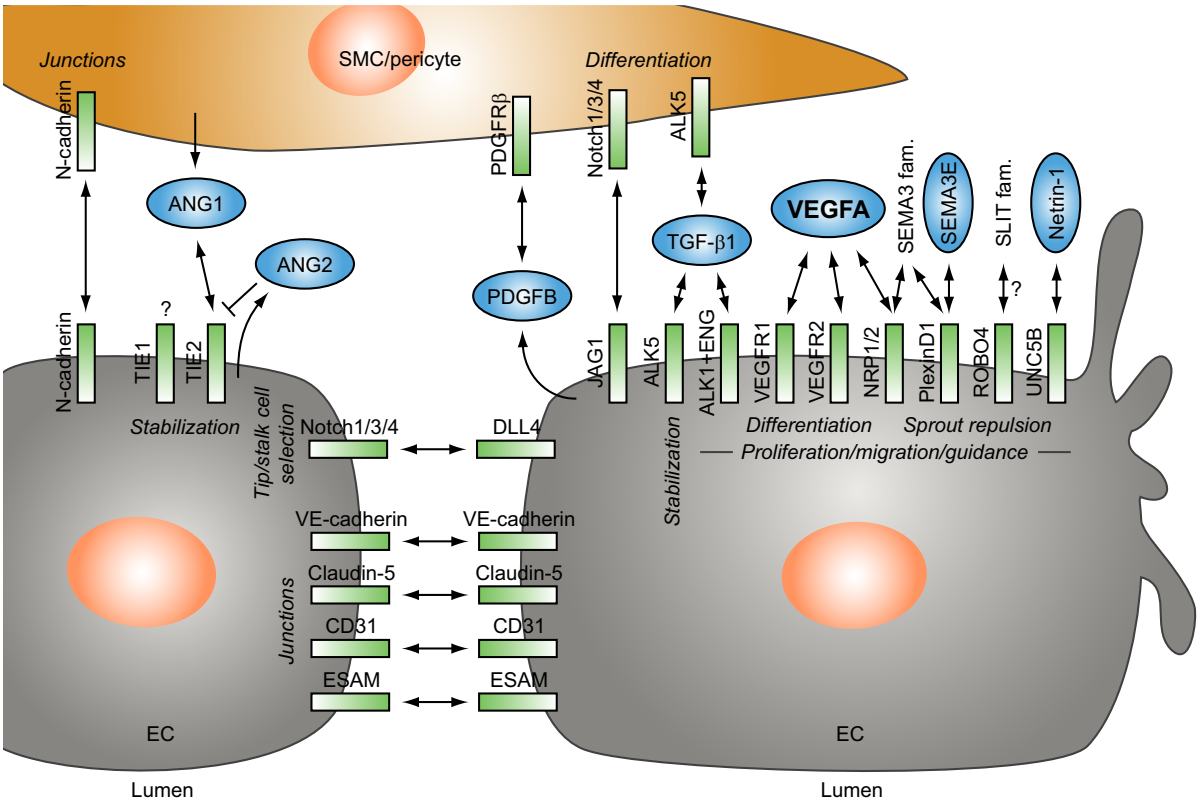


Figure 2. Basic signaling events in vascular development. Interactions that are uncertain are marked with a "?". The emphasis is on vascular-specific mechanisms; e.g. several additional proteins, with broader expression in other cell types, are important in the formation of EC-EC junctions.

overexpression in arterial ECs leads to induction of venous markers and downregulation of arterial genes [38]. In arterial cells, the Notch signaling pathway has a vital role for proper endothelial differentiation [39]. Neuropilin 1 and 2 (NRP1/2) are receptors for class 3 semaphorins, which are signaling molecules in neural development, but NRP1/2 are also capable of binding to VEGFA and other VEGF family members [40]. In blood vessels, NRP1 is expressed primarily in arteries while NRP2 is a marker for vein endothelium. Hemodynamic factors such as blood pressure and oxygenation also seem to be of importance [41].

At the tip of angiogenic sprouts is a specialized EC; the *tip cell* [42]. Tip cells extend long filopodia and guide the vascular sprout by migrating in response to VEGFA gradients. The proliferative response of VEGFA occurs only in the stalk cells that follow behind. Here too, Notch signaling has a role, as it was recently shown that the selection of which cells will become tip cells and stalk cells depends on cell-cell communication by delta-like ligand 4 (DLL4) binding to Notch receptors (Notch1/3/4). DLL4, which is membrane bound, is expressed in tip cells, and activation of Notch signaling in neighboring cells reduces expression of VEGFR2 and prohibits them from sprouting [43, 44]. Expression of VEGFR2 is therefore

most prominent in tip cells. Cell-cell interaction is also central in establishment of the *junctions* that connect neighboring cells in the endothelial monolayer. Several adhesive proteins form these contacts, many of which are also expressed by epithelial cells. Among those with EC-specific/selective expression are vascular endothelial cadherin (VE-cadherin), Claudin-5, CD31 (Pecam) and endothelial-cell selective adhesion molecule (ESAM) [45].

VEGFA is more or less dedicated to control of vascular development and can be considered the major growth factor that controls growth of blood vessels. However, in practice a multitude of guidance molecules are involved, and many of these have dual roles in vascular and neural development [24]. Among those are the class 3 semaphorins, which can control endothelial sprouting by interacting with neuropilins and receptors of the plexin family. As an example, SEMA3E appears to have a repulsive effect on vascular sprouts, since loss of its receptor plexin-D1 causes blood vessels to extend into SEMA3E-expressing somitic tissue [46]. Netrins is another class of neural guidance molecules, and Netrin-1, which binds to the endothelial-selective receptor UNC5B, has a repulsive effect on vascular sprouting [47]. In addition, ROBO4, of the roundabout receptor family which is known to interact with the SLIT family of neural guidance proteins, inhibits EC migration and is essential for normal vascular development [48, 49].

1.3.3 Mural cell differentiation and recruitment

Maturation into a functional vasculature involves differentiation and recruitment of mural cells; pericytes to capillaries and SMCs to larger arteries and veins. Several signaling pathways are implicated in the process (Figure 2). There are similarities between the two cell types; both have resemblances with fibroblasts but in contrast to SMCs, pericytes appear to have a more limited, and/or debated, contractile ability [50]. Although normally considered to be derived from surrounding mesenchymal tissue, it is clear that mural cells may have a complex developmental origin [51]. Transforming growth factor-β1 (TGF-β1), a multifunctional cytokine that is expressed by a range of cell types including ECs, is important in promoting differentiation of progenitor cells into pericytes and vascular SMCs (VSMC) [52]. TGF-β1 also has effects on the expression of important phenotypic markers in mature VSMC (see Section 1.3.5). Although TGF-β1 has a direct effect on mural cell differentiation [53-55], mural cell defects observed in knockouts of TGF-β1 or its receptors is likely to be secondary to effects on the endothelium. The TGF-β1 receptor activin receptor-like kinase-1 (ALK1) and its accessory receptor endoglin (ENG) are both selectively expressed in endothelium, and activation through this complex promotes cell proliferation and migration [56]. As a result, *Eng* and *Alk1* knockout mice both have normal vasculogenesis, but defective vascular remodeling [57, 58]. Differentiation and maturation responses to TGF-β1 are instead mediated by signaling through ALK5 [59], which has a broader expression pattern. Another

pathway of importance in mural cell differentiation is, again, Notch signaling, and it was recently shown that the Notch ligand Jagged1 (JAG1) is required on the endothelium for the development of neighboring VSMCs [60].

Proliferation, migration and recruitment of pericytes and SMCs to the vessel wall depend on platelet-derived growth factor B (PDGFB). PDGFB is most strongly expressed in the tip cells of endothelial sprouts, and it signals to PDGFRβ which is expressed by mural cells. Complete or EC-specific knockout of *Pdgfb* or knockout of its receptor *Pdgfrb* leads to various degrees of pericyte and VSMC loss, and this causes secondary effects, such as hyperplasia, on the endothelium [61-65]. Secreted PDGFB binds to the surrounding ECM through interaction between proteoglycans and a specific retention motif, and deletion of this motif also leads to reduced pericyte coverage (this transgenic model is used in **Paper V**) [66].

TIE2 (TEK) is an endothelium-selective receptor [67] and its activating ligand angiopoietin 1 (ANG1) is expressed mainly by mural cells [68, 69]. Knockout studies have revealed that this signal is critical for vessel stabilization and maturation; mice null for the receptor or the ligand have reduced pericyte coverage and defective angiogenesis. In addition, overexpression of ANG1 can partly rescue vessels that have been made leaky by e.g. inhibition of PDGFR-β [70, 71]. ANG2 is a related factor expressed mainly by ECs that has an antagonistic effect on the TIE2 receptor. Overexpression of this protein causes angiogenesis defects similar to the ANG1/TIE2 knockouts [72], while knockout does not disturb embryonic angiogenesis [73]. Interestingly, in adult tissues, ANG2 appears to be expressed primarily at sites of active vascular remodeling, suggesting that its role may be to block the stabilizing function of ANG1 in sprouting vessels [72]. This is supported by results suggesting that tumor-derived VEGF upregulates ANG2 in ECs of the stroma, supposedly to destabilize and facilitate remodeling of the host vasculature [74]. The role of TIE1, an endothelium-specific receptor closely related to TIE2, is not fully understood. TIE1 deficiency in mice leads to vascular defects and late embryonic lethality [67], but it is still unclear which ligands can activate this receptor [75].

The actual contact between ECs and mural cells is believed to trigger a more stable, non-sprouting phenotype [52]. ECs and pericytes are connected through adherence junctions based on N-cadherin, which is expressed both by ECs/mural cells but also neural cells [76, 77].

1.3.4 The vascular smooth muscle cell

Smooth muscle, so-called because it lacks the regular striated appearance of skeletal and heart muscle, provides contractility to a range of hollow organs, such as the stomach, the urinary bladder and blood vessels. SMCs have an elongated shape and are arranged in ordered layers, where mechanical coupling is provided by adherens

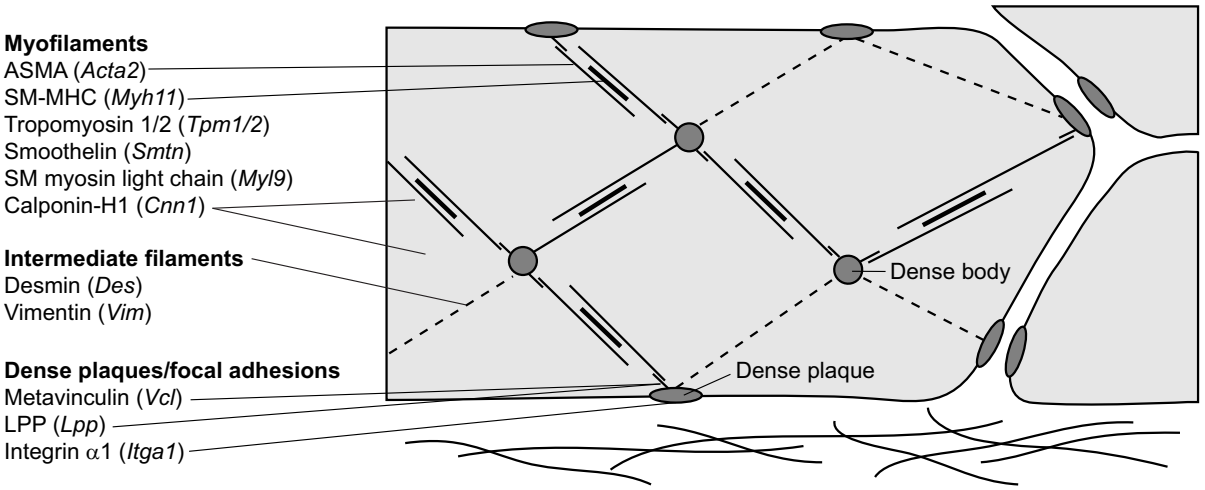


Figure 3. Some established smooth muscle markers in the context of the SMC cytoskeleton. Positions essentially follows descriptions in [80]. It should be noted that while some of these genes are highly restricted to SMCs, others have additional expression in other locations. Intermediate filaments are not contractile but are thought to play a mechanical role in force transmission [114]. Knowledge of smoothelin function is still incomplete, but it has been suggested to be positioned in the contractile apparatus [115]. Calponin-H1 localizes both to the contractile machinery and to the cytoskeleton [116]. LPP has been shown to colocalize with vinculin, a linker protein, at points of cell-matrix or cell-cell contact (dense plaques/focal adhesions) [117]. Mouse gene symbols are shown within parentheses.

junctions between neighboring cells. Like in striated muscle, contraction is triggered in response to increased intracellular Ca^{2+} concentration [78]. However, in SMCs the action is considerably slower, but also very economical (several hundred-fold more efficient compared to skeletal muscle [79]) and can achieve a much higher degree of shortening. By providing contractile properties to the vascular wall, SMCs enable control of blood pressure and blood distribution within the body. In addition, SMCs have an important role in synthesis of extracellular matrix (ECM) in the vessel media. SMCs express a number of differentiation marker genes, many of which encode smooth muscle isoforms of contractile proteins [80]. These include smooth muscle α-actin (ASMA, *Acta2*), smooth muscle myosin heavy chain (SM-MHC, *Myh11*) and LPP (*Lpp*) (the transcriptional regulation of LPP is investigated in **Paper II**). Figure 3 illustrates the function and location within the SMC cytoskeletal structure for a number of those genes.

SMCs are considered to be *phenotypically plastic*, meaning that they can undergo drastic changes in function and appearance in response to environmental signals [12, 81]. This is important during vascular development, when SMCs show a high rate of proliferation, migration and synthesis of ECM components ("synthetic phenotype"). In comparison, quiescent SMCs in mature blood vessels have a very low rate of proliferation and synthetic activity, i.e. matrix production, while contractile proteins are expressed at high levels ("contractile phenotype"). The idea of contractile vs. synthetic/proliferative phenotypes as opposite states is a

simplification and is also challenged by observations from large-scale transcription profiling experiments [82]. Consequently, the concept of phenotypic switching is also used in a broader sense to describe more subtle changes in various functional characteristics of SMCs. Phenotypic modulation/switching of SMC function is involved in a number of vascular disorders including hypertension [83] and atherosclerosis [84]. A key feature of atherosclerotic plaques is the accumulation of SMCs within the intima. These cells are generally considered to be phenotypically modulated SMCs that migrate from the media, but contribution from circulating progenitor cells [85] or transdifferentiation of endothelial cells has also been suggested [86]. Intimal SMCs are in a synthetic state and contribute to the formation of the fibrous cap that encapsulates the plaque. At least in the later stages of the disease, they are therefore likely to have a beneficial role in that they stabilize the plaque and help to avoid rupture [87]. An interesting view that challenges the idea of phenotypic modulation is the possibility that predisposed SMCs, which can contribute to intimal thickening, are already present among a heterogeneous population within the media [88].

1.3.5 Regulation of smooth muscle differentiation markers

Significant effort has been put into understanding the transcriptional regulation of SMC differentiation markers. SRF, together with various cofactors, has emerged as central in activation of these genes [12, 51, 81, 89]. SRF, a member of the MADS box family of transcription factors [90], recognizes a specific 10 bp *cis*-element, the CArG box/SRE[1] ($CC[AT]_6GG$). CArG boxes are present in the majority of SMC markers, and those that lack a CArG box, such as ACLP [91], generally appear to be of the category that has less specific expression [89]. SMC genes may have a single CArG (e.g. telokin or desmin) or multiple CArGs (e.g. SM-MHC or ASMA). These are always located within a few thousand base pairs from the TSS, sometimes in intronic positions. In many cases, paired CArG boxes are located relatively close to each other, and this raises the possibility of cooperative interaction between them. The ASMA promoter contains two CArGs separated by 40 bp, and this organization is conserved over a range of species. Interestingly, introduction of a 5 or 15 bp spacer in between the two inhibits promoter activity, while insertion of 10 bp or 20 bp spacers retains a lot of the activity [92]. This indicates that the "phase" of the DNA helix, which has a periodicity of ~10 bp, affects interaction between the two sites (we developed a motif discovery tool, HeliCis, which incorporates this type of spatial relationship [93]).

Although SRF is highly expressed in smooth muscle, it is far from SMC-specific as it is also vital for e.g. development of heart and skeletal muscle [94, 95]. Consequently, SRF alone cannot active transcription specifically in SMC. SRF can

[1] SRE, serum response element, was originally used to describe a longer SRF-binding element, present in early-response genes such as *c-fos*, but is often used interchangeably with "CArG-box".

interact with a number of proteins, including GATA4 and Nkx2.5 in activation of cardiac-specific genes and MyoD and myogenin in skeletal muscle [89, 96]. In smooth muscle, the myocardin family of SRF cofactors has a central role in activation of SMC marker genes [96, 97]. The family includes myocardin-related transcription factor A (MRTFA, Mal, MKL1) and MRTFB (MKL2) and myocardin. Myocardin does not have DNA binding capability of its own, and transactivation of SMC markers by myocardin involves direct interaction with the SRF-CArG complex. In addition to SMC, myocardin is also expressed in heart where is activates cardiac genes together with SRF [98]. The potency of myocardin in activation of SMC transcription is demonstrated by overexpression experiments in a number of different non-SMC cell lines; overexpression of myocardin in e.g. ES cells induces expression of SMC differentiation markers in an SRF-dependent fashion [99]. MRTF-A/B seem to have similar activating capabilities as myocardin, but their expression patterns are less restricted [96]. While both myocardin and MRTFB deficiency leads to embryonic lethality due to smooth muscle defects [100, 101], MRTFA-null mice appear to have normal vasculature [102].

Recent data suggests that myocardin can activate SMC gene expression in skeletal muscle progenitor cells (myoblasts) in mice, while at the same time repressing expression of the skeletal muscle differentiation program through repression of myogenin [103]. The majority of skeletal progenitors transiently express myocardin at some point during differentiation, and the results indicate that myocardin acts as a bifunctional switch that has the capacity to convert skeletal myoblasts to a SMC phenotype. Further support comes from experiments showing that myocardin overexpression in a skeletal muscle progenitor cell line induces a SMC-like phenotype, while at the same time causing attenuation of myogenin [104].

In addition to myocardin, a large number of transcriptional activators and repressors contribute in various ways to SRF-dependent expression of SMC markers. Activators include CSRP1/2, SMAD2/3, KLF5, PIAS1, ATF6 and NF-κB, and repressors include YY1, KLF4, ELK1, SP1/3, HOP, FHL2 and HRT2 [12, 51]. Among other things, these mediate responses to external stimuli that modulate the SMC phenotype, such as TGF-β1 and PDGFB. TGF-β1 increases expression of most SMC differentiation markers, and this has been shown to be mediated by TGF-β control elements (TCE) in the promoters of several SMC genes [105]. Transcription factors of the Kruppel-like family can bind to the TCE; e.g. KLF5 appears to have an activating function and KLF4 represses this element [106]. Consistent with its role in vessel maturation (Section 1.3.3), PDGFB appears to modulate SMC towards a more "synthetic/proliferative" state. Stimulation by PDGFB drastically suppresses SMC marker expression in cultured SMC [107] and neointimal formation in atherosclerosis can be reduced by inhibition of PDGFRβ in a mouse model of atherosclerosis [108]. Although several mechanisms seem to

contribute [109], PDGFB-induced suppression of SMC genes has been shown to be mediated by the ETS-domain factor ELK1 [110]. ELK1 is phosphorylated in response to PDGFRβ activation, and phosphorylated ELK1 can compete with SRF for binding to CArG elements.

1.3.6 Regulation of endothelial differentiation markers

Like SMCs, ECs express a unique set of phenotypic markers (many of which are reviewed in Section 1.3.2) which encode core proteins required for endothelial cells to function within their environment. Forkhead box factors (FOXs) such as FOXC1/2 and ETS factors such as FLI1 are known to be of importance in endothelial differentiation, and binding sites for ETS factors (GGA[AT]) are present in nearly all EC-specific promoters [111]. A multitude of different ETS factors that can bind to these sites are present in ECs, and none of them have an endothelium-restricted expression pattern. Moreover, these sites are not specific to EC promoters. In addition, a large number of other transcription factors that each act on a limited number of endothelial genes have been identified, but a master switch similar to that of SRF/myocardin in SMCs has been considered lacking in ECs [112]. However, recently the first report of a common regulatory mechanism for EC-specific gene transcription was published [113]. In this study, a 44 bp transcriptional enhancer that is sufficient to direct expression specifically to ECs was identified. This enhancer contains a composite regulatory element, the FOX:ETS motif, which is present in the regulatory regions of several established EC markers, including *Kdr*, *Tie2*, *Notch4* and *Cdh5*. Although several Forkhead and ETS factors may be involved, it was specifically shown that FOXC2 and ETV2 can bind to this element, and that combined overexpression of these two factors induces transcription of EC-specific genes in normally avascular regions.

2 Objectives

Improved understanding of the molecular mechanisms that govern blood vessel development and function is key to development of future therapies that target vascular-related pathological conditions. Previous studies have revealed that genes expressed specifically in EC and SMC have vital roles in the vasculature. The overall aim of this thesis has been to make use of genome-wide methods and datasets to expand our knowledge of vascular-specific gene expression and gene regulation, and to investigate how genetic variation in these genes contributes to human disease.

More specifically, the objectives were:

- To investigate if cases of transcriptional coregulation in mammalian cell differentiation could be detected using computational methods and publicly available sequence and expression data (**Paper I**)
- To study the regulation of one specific SMC marker gene (LPP) and investigate if it was controlled by similar regulatory mechanisms (SRF/myocardin) as other SMC markers (**Paper II**)
- To increase our knowledge of specific gene expression in the endothelium through identification of novel EC markers (**Paper III**)
- To investigate if genetic variation in EC-specific genes contributes to human disease (**Paper IV**)
- To discover miRNAs that are preferentially expressed in the microvasculature, and to investigate their role in regulation of vascular gene expression (**Paper V**)

3 Results and discussion

3.1 Modular regulation in the mammalian genome (Paper I)

3.1.1 Functionally coupled coexpression clusters

We developed a computational procedure to screen the mouse and human genomes for occurrences of gene battery-like gene regulation, i.e. coexpressed sets of genes activated by common transcriptional regulators. To accomplish this, we made use of three types of data: the mouse and human genome sequences, descriptions of transcription factor binding motifs in the form of position frequency matrices (PFM) from the TRANSFAC [118] and JASPAR [119] databases and microarray expression data from the Novartis SymAtlas; a compendium of gene expression profiles covering 140 normal tissues/cell types in mouse and human [120]. A schematic overview the procedure is shown in Figure 4.

The analysis was based on the assumption that human and mouse homologous genes (orthologs) are regulated in similar ways. Reciprocal human and mouse ortholog pairs were identified using the ENSEMBL database [1] and coexpressed gene groups, with genes having similar expression profiles over a wide range of tissues in mouse and human, were identified using agglomerative hierarchical clustering [121]. This generated a set of 160 clusters containing a total of 2407 ortholog pairs. Manual inspection revealed a strong functional coupling within these coexpression clusters, and unbiased statistics showed that the grand majority of the larger clusters were statistically enriched for one or several functional annotation terms (gene ontology; GO). The identified clusters contained genes that represented specific tissues (e.g. liver-specific genes) and molecular processes (e.g. subunits of the ribosome), but also cell types. As an example, the smooth muscle differentiation markers *Actg2*, *Myh11*, *Lpp*, *Tagln*, *Acta2*, *Myl9* and *Lmod1* formed one cluster (Figure 5A). Endothelial marker genes were found to have a distinct expression profile characterized by high expression in the lung. As a result, several known EC markers (*Cdh5*, *Tie1* and *Vwf* and others) clustered together with genes coding for e.g. lung surfactant proteins. In **Paper III** we explore this coexpression group further and combine it with in-house expression data to make an accurate prediction of genes with specific/selective expression in the endothelium.

3.1.2 Linking clusters to regulators

Coexpression clusters were further evaluated with respect to shared transcriptional regulators. Prediction of *cis*-regulatory elements is complicated by the fact that sequence motifs recognized by transcription factors commonly have low information content, i.e. they have a high probability of appearing by chance in random, non-functional DNA. A technique that is often used for reducing false

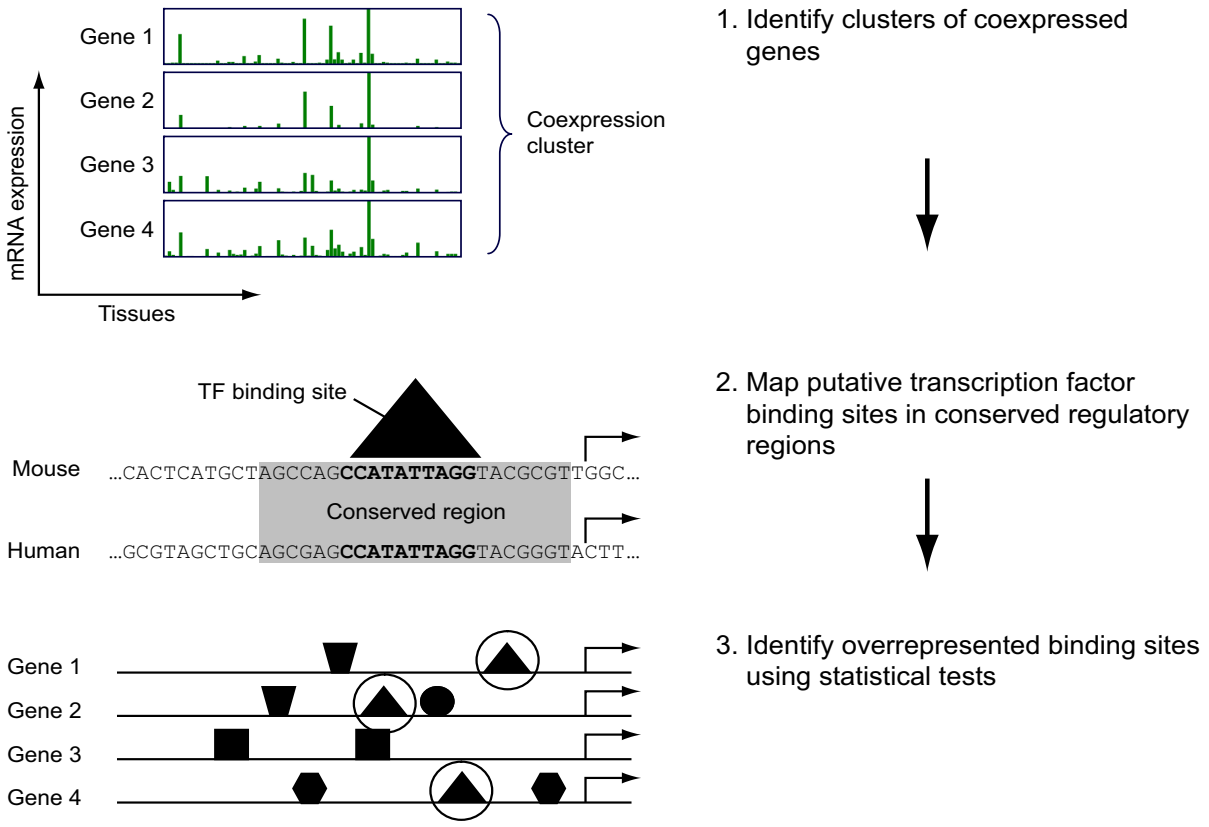
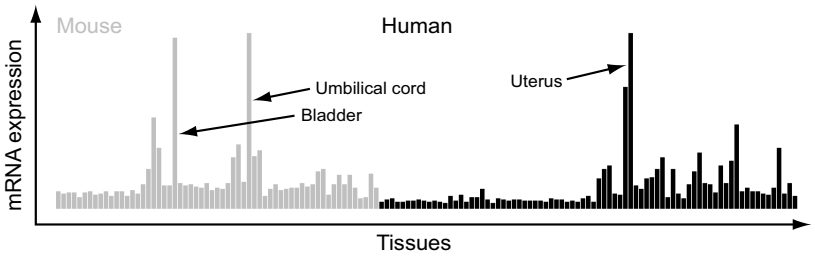


Figure 4. Overview of the computational procedure used to identify common *cis*-regulatory elements in groups of coexpressed genes.

positive predictions is *phylogentic footprinting*; masking of regions that are not evolutionarily conserved [122]. The rationale is that functional DNA tends to be under positive evolutionary selection and as a consequence, true TF binding sites are often localized in areas of sequence conservation. For each gene in the dataset, regions of DNA proximal to the TSS that showed evolutionary conservation between mouse and human were identified. Putative transcription factor binding sites were mapped onto these regions and statistical tests were used to detect motifs that were overrepresented in specific clusters relative to remaining genes in the dataset.

At a false discovery rate (FDR) of 10%, 66 motifs could be associated to 21 different clusters using this method. The overrepresented motifs represented both previously described cases of gene battery-like regulation, novel cases of gene battery-like regulation and novel cases with some support in a limited number of genes. In the case of the smooth muscle cluster, regulation by SRF was rediscovered at a high level of statistical confidence (Figure 5B). In addition, YY1, a known regulator of muscle-specific gene expression that acts through inhibition of SRF at CArG boxes [123], and TEF-1, known to regulate smooth muscle-specific genes through binding to MCAT elements [124], were associated to this battery,

A



B

PWM	Cluster coverage	Dataset coverage	FDR
SRF	86% (6/7)	1% (89/9561)	<2.5%
SRF	86% (6/7)	1% (114/9561)	<2.5%
SRF	71% (5/7)	1% (135/9561)	<2.5%
TATA	43% (3/7)	2% (220/9561)	<20%
YY1	43% (3/7)	3% (284/9561)	<20%
TEF-1	43% (3/7)	3% (296/9561)	<20%

Mouse symbol	Human symbol	Description (mouse)
Actg2	*ACTG2*	ACTIN, GAMMA 2, SMOOTH MUSCLE, ENTERIC.
Q8C3J0	*MYH11*	MYOSIN HEAVY CHAIN 11.
Lpp	*LPP*	Lpp LIM DOMAIN CONTAINING PREFERRED TRANSLOCATION PARTNER IN LIPOMA.
Tagln	*TAGLN*	TRANSGELIN (SMOOTH MUSCLE PROTEIN 22-ALPHA) (SM22-ALPHA) (ACTIN- ASSOCIATED PROTEIN P27).
Acta2	*ACTA2*	ACTIN, AORTIC SMOOTH MUSCLE (ALPHA-ACTIN 2).
Myl9	*MYL9*	MYL9 PROTEIN (FRAGMENT).
Lmod1	*LMOD1*	LEIOMODIN 1 (SMOOTH MUSCLE); 64KD D1.

Figure 5. Smooth muscle differentiation cluster. (A) The bar graph shows the average expression level of genes in the cluster across a range of tissues in mouse (gray) and human (black). (B) Transcription factor binding site motifs that are statistically overrepresented within the cluster. FDR, false discovery rate.

although at a low level of significance. Interestingly, the analysis indicated that the *Lpp* gene could be regulated by SRF. Earlier work within the group identified *Lpp* as a smooth muscle marker gene [125] and a later study revealed a potentially important function for the LPP protein in vascular disease due to its role in control of SMC migration [126]. However, little has been known about the regulation of this gene, and we further investigate the hypothesis that SRF regulates *Lpp* in **Paper II**.

3.1.3 Novel regulatory mechanisms

The SMC example, and several other established regulatory mechanisms that were rediscovered in the present work, gives confidence to the novel cases of coregulation that were identified. One finding with possible implications for vascular biology was the enrichment of the PAX4 motif in a cluster of genes containing components and modulators of the extracellular matrix. The expression pattern of genes in this cluster is characterized by strong signal in highly vascularized tissues and many of the corresponding proteins are known to be synthesized by SMCs in the vessel media. However, since there is rarely a one-to-one relation between motif and binding factor, there is a need for careful interpretation of such results. As an example, the MEF2 motif, which we correctly identified as a regulator of cardiac and skeletal muscle specific genes, can bind to several factors of the MEF family (MEF2A, MEF2B, MEF2C and MEF2D) [127].

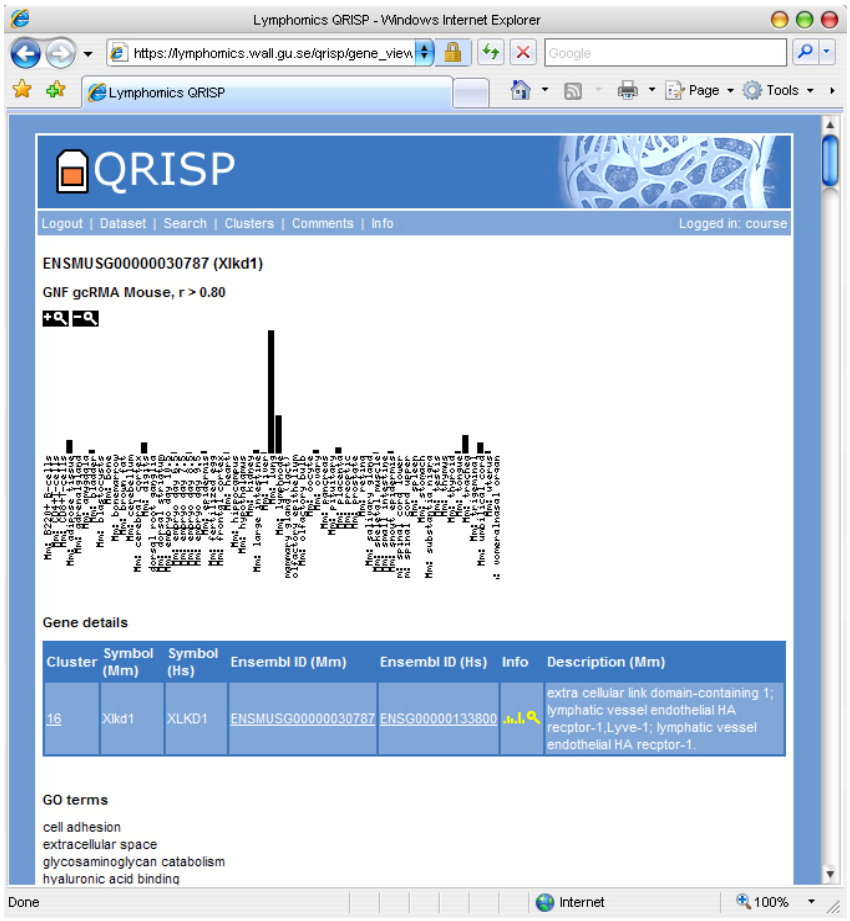


Figure 6. Screenshot of the QRISP database. QRISP is a web-based application that can be used e.g. to identify coexpressed genes and coexpression clusters, and to discover shared transcription factor binding sites within such clusters.

It is therefore likely that several regulatory proteins can recognize the GC-rich [128] PAX4 motif. In fact, further analysis revealed the Krüppel-like GC-rich-binding transcription factor Zfp148 (Zbp-89) [129] to be a better match for those sites (unpublished data).

In the case of the SMC battery, 6/7 (86%) of the ortholog pairs were predicted to contain an SRF binding site. However, in the typical case, coverage was considerably lower, the average being 15%. This may indicate that other mechanisms than shared *cis*-regulators contribute to coordinated expression in these clusters. However, coverage is also likely to be reduced by a number of technical factors, one being that functional regulatory elements can be present outside of the limited amount of sequence that was used in the analysis (2 kb upstream for the final analysis, although larger regions were also evaluated).

In conclusion, through computational analysis of publicly available biological datasets we identified 21 instances of statistically supported gene battery-like regulation in mammals, and these included both known and novel cases. Results suggested that the smooth muscle marker LPP could be regulated by SRF and this is investigated in detail in Paper II. The computational principles that were used in

this study were further developed into a web-accessible application, the QRISP database [130] (unpublished, Figure 6).

3.2 LPP is regulated by SRF (Paper II)

Previous work in our group [125] and elsewhere [117] identified lipoma preferred partner (LPP) as a SMC marker. In cultured aortic SMCs and fibroblasts, LPP localizes to focal adhesions [117, 131, 132] but it can also shuttle to the nucleus and function as a transcriptional coactivator [133]. LPP is expressed in neointimal SMCs and controls SMC migration downstream of focal adhesion kinase (FAK) [126]. This suggests that LPP may play an important role in vascular disease, and the mechanisms by which LPP are regulated in physiological and pathological conditions are therefore of clinical interest. Many smooth muscle markers are activated by SRF and its cofactors, and results from **Paper I** indicated the presence of a CArG box (SRF binding site) in the *Lpp* gene. Previous work showed that overexpression of the SRF cofactor myocardin increases *Lpp* mRNA expression [126]; however, regulation of LPP by SRF remained an open question. We therefore sought to investigate whether the *Lpp* gene was directly regulated by SRF in a CArG-dependent fashion.

3.2.1 A conserved CArG in an alternative promoter

The mouse *Lpp* gene spans a large genomic region of nearly 600 kb. To identify possible SRF binding sites, we used proprietary software to scan the complete *Lpp* locus plus flanking sequence for putative CArG boxes. This lead to the identification of 35 possible sites, of which three (8, 11 and 13) also showed strong evolutionary conservation over a range of species (Figure 7). These were all located far from the TSS (55-100 kb). A large proportion of mouse and human genes have alternative TSS [134, 135] and we therefore chose to screen the *Lpp* gene for alternative starts. Interestingly, several cDNA clone sequences in the DTBSS database [136], a collection of full-length cDNA sequences created in a way that ensures inclusion of the 5'-end (oligo-capping [137]), indicated presence of an alternative first exon (hereafter denoted exon 2b) in intron 2, 140 bp downstream of CArG 8 (Figure 8). Sequence analysis indicated the presence of a basal promoter that coincided with this TSS and the presence of a proximal promoter was further indicated by the fact that a 250 bp region upstream of the alternative first exon showed strong evolutionary conservation across 10 mammalian species. Presence of the alternative transcript could be confirmed using reverse transcription PCR (RT-PCR) in SMC-containing tissues.

3.2.2 The CArG-containing promoter activates SMC transcription

By designing quantitative RT-PCR (qRT-PCR) assays that specifically targeted the alternative transcript or the normal transcript, the tissue specificity of the two

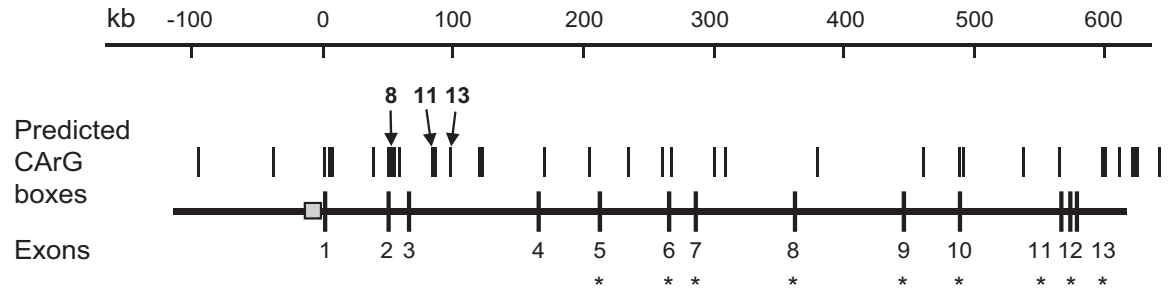


Figure 7. Putative CArG boxes in the mouse Lpp gene including +/- 100 kb of flanking sequence.

promoters could be investigated. The alternative CArG 8-containing promoter was active in SMC-containing tissues and showed an expression pattern that was highly similar to *Myh11*, which is considered to be the most specific SMC marker [138], while the upstream promoter exhibited a ubiquitous expression pattern. Although the alternative promoter could therefore concluded to be responsible for expression in SMCs, the role of the predicted CArG box (CArG 8) needed to be determined. Chromatin immunoprecipitation (ChIP) experiments showed binding of SRF to endogenous DNA in this region in cultured primary aortic SMCs, and electrophoretic mobility shift assay (EMSA) experiments further confirmed that SRF could bind to the predicted site. To validate a functional role for SRF in activation of this promoter, luciferase reporter experiments were performed. A ~500 bp fragment containing the alternative TSS and the conserved upstream region, including CArG 8, was cloned into a promoter-less luciferase vector (pGL3basic). For comparison, similar vectors were designed from the CArG 11 and 13 regions. The CArG 8-containing construct showed strong activity compared to the other constructs, while removal of the CArG box reduced the signal by 75%. Overexpression experiments revealed the CArG 8-containing promoter to be responsive to SRF and myocardin, while the ΔCArG mutated construct was not. Unexpectedly, increasing amounts of SRF caused a decrease in reporter activity in this system. This has previously been described for SRF in the case of the *C-fos* promoter [139] and can likely be attributed to a phenomena referred to as squelching; sequestering of coactivators.

3.2.3 SRF regulates the alternative transcript *in vivo*

To confirm that the endogenous transcript produced by the CArG 8-containing promoter was responsive to SRF, we examined whether expression was affected by SRF overexpression or knockout in cell lines and transgenic mice. Levels of the alternative transcript were found to be reduced by 70% in an SRF-deficient embryonic stem cell (ES) line [140] compared to wild type ES cells. In contrast, expression of the upstream transcript was unaffected. Overexpression of a constitutively active SRF-VP16 fusion protein [141] in these cells caused a ~10-fold

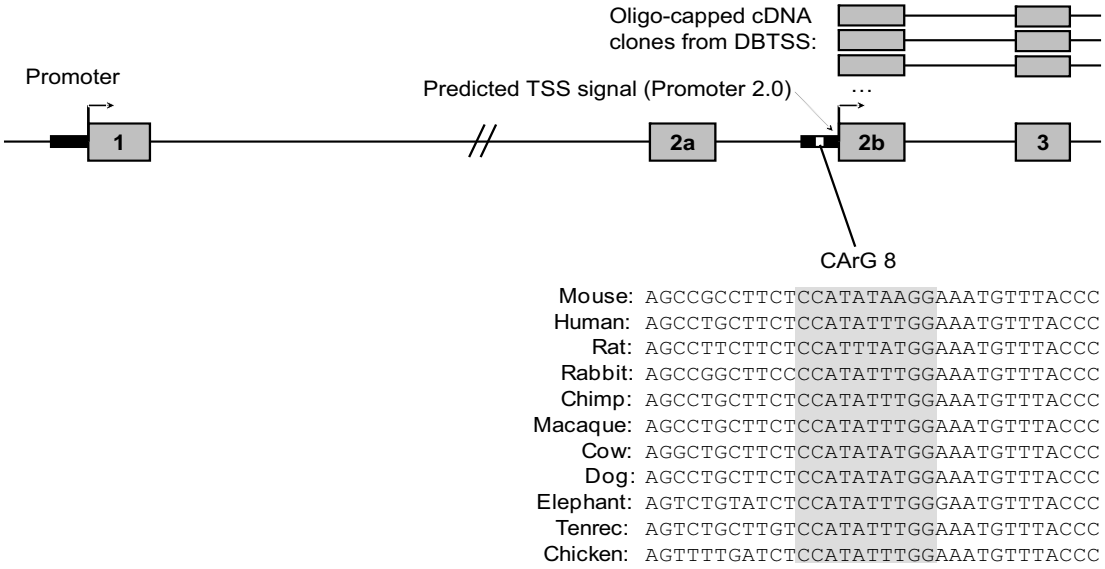


Figure 8. Schematic showing exon 1-3 of the Lpp gene. cDNA sequence evidence indicates the presence of an alternative transcription start site (TSS) and first exon (exon 2b) in intron 2. The alternative start coincides with a putative basal promoter predicted using the Promoter 2.0 software. The alternative TSS is preceded by a strongly conserved CArG box (CArG 8).

increase in expression of the alternative transcript, while only a small effect was seen on the upstream transcript.

SRF-deficient mice die during early embryogenesis due to failed mesoderm formation [142], and studies of SRF deficiency in adult animals consequently require more sophisticated models. We investigated transcript levels in mice homozygous for a floxed *Srf* allele (Srf^{flex1}) crossed with mice expressing an inducible Cre recombinase under control of the *Tagln* (SM22α) promoter [143]. This model ($Srf^{flex1/flex1}$:$SMCreER^{T2}/wt$) allows inducible SMC-specific deletion of SRF in adult animals. mRNA levels of the alternative *Lpp* transcript were found to be reduced by 75-90% in smooth muscle containing tissues of SRF knockout mice, while levels in the heart were unchanged as expected. The upstream transcript was also affected but to a lesser extent. This showed that activity of the alternative promoter in SMCs is controlled by SRF *in vivo*.

The present study revealed that SRF/myocardin regulates *Lpp* transcription through binding to an evolutionarily conserved CArG box in an alternative promoter, located in intron 2. This promoter produces a transcript in which exon 1 is replaced by an alternative first exon. The open reading frame of this transcript is however unchanged and its main purpose is therefore likely to promote transcription of *Lpp* in SMCs. Interestingly, we found only a single CArG box in the alternative promoter of intron 2, while many SMC marker genes contain a cluster of two or more closely spaced CArG boxes (Section 1.3.5). This property

has been proposed to promote homodimerization and activation of myocardin [97], therefore making it unable to activate transcription of non-muscle SRF targets such as *c-fos*, which contain only a single CArG [144]. However, the SMC marker telokin, a product of a downstream alternative promoter in *Mlck* gene, contains only a single CArG and is still myocardin-responsive, even using a dimerization-defective mutant myocardin construct [145]. In conclusion, our data adds *Lpp* to the list of smooth muscle marker genes regulated by SRF and myocardin.

3.3 Novel endothelial marker genes (Paper III)

Like SMCs, ECs express their own set of cell-type specific effector genes (see Section 1.3.2). These genes represent important core functions of the endothelium, such as the VEGF receptors (e.g. *Kdr* and *Flt1*) or *Notch4*. Several of those, or their associated ligands, are being considered for therapeutical targeting in the treatment of diseases where the angiogenic process plays an integral part, such as cancer and age-dependent macula degeneration (AMD) [146-148]. Identification of novel microvascular-specific genes can reveal new targets and provide clues for future therapies.

3.3.1 Combining public and in-house data

Work done in **Paper I** revealed that established endothelial marker genes have an expression profile characterized mainly by high expression in the lung (Figure 9 shows expression of *Kdr* (VEGFR2) and *Tie1* over a range of tissues in the SymAtlas dataset [120]). It is clear that by screening for genes having expression profiles similar to known endothelial markers over a range of tissues, new specific or selective genes can be discovered. However, such a list will also be contaminated by markers of lung epithelium, such as the pulmonary surfactant proteins (e.g. *Sftpb, Sftpc and Sftpc*) (Figure 9). To segregate the predictions into true endothelial markers vs. lung epithelial markers, we combined our results with in-house Affymetrix microarray expression data from the *kidney glomerulus* [149]. By perfusion with magnetic 4.5 μm Dynabeads, glomeruli can be separated from the remaining kidney with high yield and minimal contamination of non-glomerular cells [150]. Glomeruli have a high density of endothelial cells, but represent only a small proportion of the total kidney mass. Known endothelial markers consequently display a high degree of differential expression in glomeruli vs. the remaining kidney. The two datasets, created on different technical platforms, were merged by re-annotation using BLAST [151] against the ENSEMBL collection of transcripts and gene sequences [1]. This combined analysis allowed identification of genes with broad and specific expression in the microvasculature and this would not have been possible using any of the two datasets alone: differential expression in the glomerulus says little about the expression pattern in other tissues and could also imply expression in glomerular epithelium (podocytes), while the tissue panel

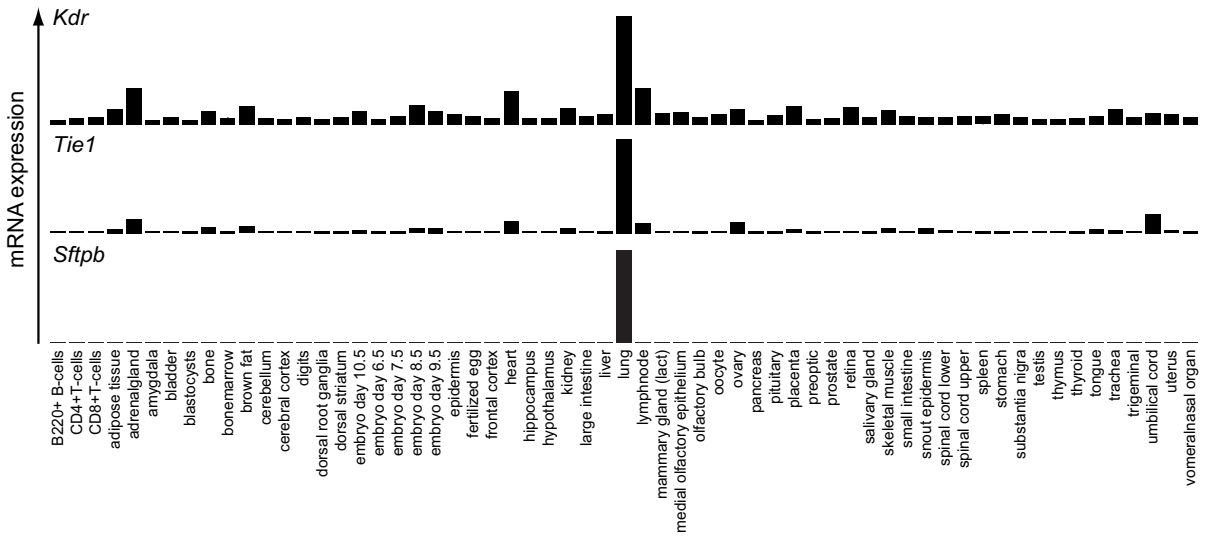


Figure 9. Transcriptional profiles over a range of normal tissues in the Novartis SymAtlas dataset. Two established endothelial markers, *Kdr* and *Tie1*, and one lung epithelium-specific gene, *Sftpb*, are shown.

analysis provided a prediction list that was contaminated by markers of lung epithelium.

3.3.2 Discovery of 32 novel microvascular markers

As can be seen in Figure 10 (a two-dimensional scatter plot of 13341 genes), the introduction of a second dataset efficiently separated lung epithelium markers from true endothelial markers. By applying thresholds for similarity to known markers and for differential expression in the glomerulus, a cluster of 71 genes that included essentially all well-established microvascular marker genes could be identified. We further validated and filtered our results against independent microarray data on microvascular fragments isolated from the mouse brain using antibody-coated magnetic beads, leaving a list of 58 genes that were likely to be both microvascular specific/selective and have broad expression in the microvasculature (Table 1). Among these were 32 genes for which we could find no published evidence of vascular-specific expression. A subset of seven genes was validated using qRT-PCR in E18.5 brain microvessels compared to surrounding brain tissue. These were all found to be microvascular-enriched (*Eltd1*, 254-fold; *Gpr116*, 1187-fold; *Ramp2*, 114-fold; *Slc9a3r2*, 87-fold; *Slc43a3*, 15-fold; *Rasip1*, 53-fold; *Hig2*, 3-fold). In addition, one gene, *Slc9a3r2*, was validated using immunohistochemistry. *Slc9a32r* showed strong and specific endothelial expression in eight normal human tissues and in tumor endothelium.

Studies in mice have shown that targeted disruption of established endothelial markers, such as *Kdr*, *Flt1* and *Cdh5*, normally leads to severe vascular development defects and embryonic lethality [30, 152-154]. The majority of genes of this

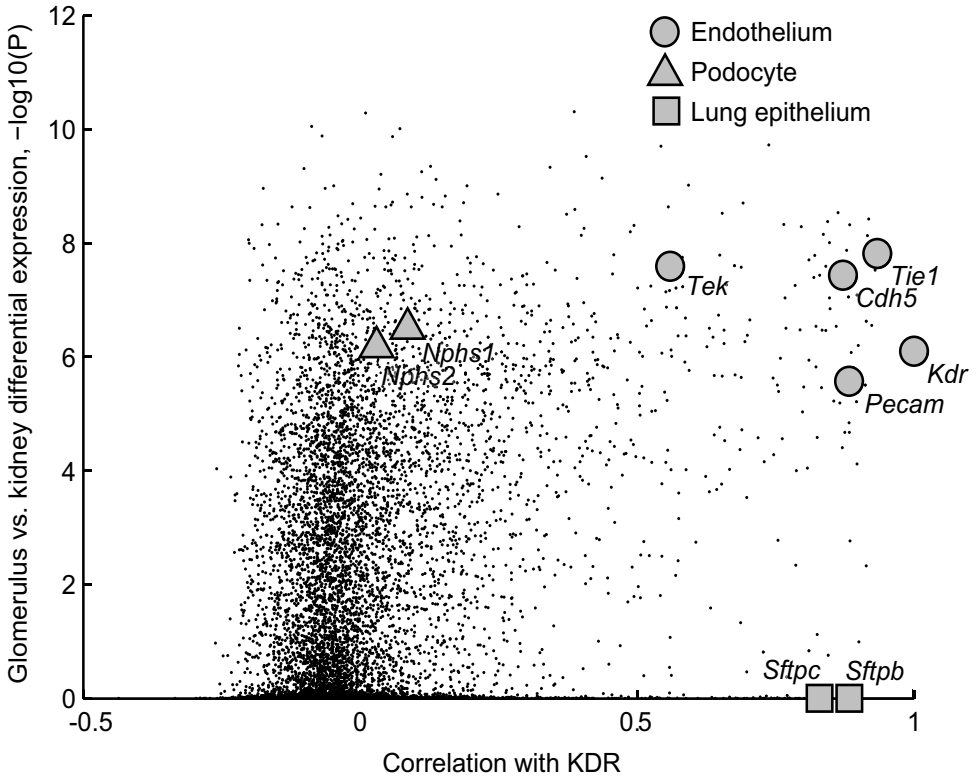


Figure 10. Identification of novel endothelial markers. The scatter plot shows similarity to a known endothelial marker (*Kdr*, x-axis) versus the degree of differential expression in the glomerulus versus the remaining kidney (y-axis) for 13341 genes. Similarity was determined by calculating the Pearson correlation coefficient in the SymAtlas tissue panel dataset. Circles, triangles and squares represent endothelium, podocyte and lung epithelium marker genes, respectively.

category are present among the 58 genes that were identified in the present study. It is therefore likely that several of the 32 novel markers have essential roles in vascular development. During the course of the present investigation, a mouse knockout study of one of the novel genes, receptor activity-modifying protein 2 (*Ramp2)*, was published, and although a function in the endothelium was previously known for this protein, the authors report here for the first time an essential role in development of the vasculature [155]. Likewise, endothelial-specific deletion of calcitonin receptor-like receptor (*Calcrl*, Table 1), which associates with *Ramp2* to form a receptor for adrenomedullin [156], resulted in abnormal development of the lymphatic vasculature due to reduction in endothelial cell proliferation [157]. Two of the novel endothelial markers that were identified, *Eltd1* (ETL) [158] and *Grp116* [159], are GPCRs for which no published functional data is available. These, and several other predicted genes, represent possible drug targets for e.g. antiangiogenic theraphy.

In conclusion, we used public and in-house genome-wide expression data to identify 58 genes with specific and broad expression in the microvasculature. This set included most of the currently known endothelial markers, but also a large

Gene symbol	Description	PubMed
Acvrl1	Serine/threonine-protein kinase receptor R3 precursor	88
Adcy4	Adenylate cyclase, type IV	0
Afap1l1	actin filament associated protein 1-like 1	0
Arap3	ARF-GAP, RHO-GAP, ankyrin repeat and pleckstrin homology domains-containing protein 3	0
Calcrl	Calcitonin gene-related peptide type 1 receptor precursor	4
Caskin2	cask-interacting protein 2	0
Ccbp2	Chemokine binding protein 2	1
Cdh5	Vascular endothelial-cadherin precursor	7
Cldn5	Claudin-5	41
Col4a3	procollagen, type IV, alpha 3	5
Cttnbp2nl	CTTNBP2 N-terminal like	0
Dram	Damage-regulated autophagy modulator	0
Dtr	Heparin-binding EGF-like growth factor precursor	4
Egfl7	vascular endothelial statin isoform 1 precursor; NEU1 protein; vascular endothelial-statin	18
Ehd4	EH-domain containing protein 4	0
Eltd1	EGF, latrophilin seven transmembrane domain containing protein 1 precursor	1
Eng	Endoglin precursor	676
Entpd1	Ectonucleoside triphosphate diphosphohydrolase 1	7
Epas1	Endothelial PAS domain protein 1	22
Ephb4	Ephrin type-B receptor 4 precursor	69
Erg	Transcriptional regulator ERG	147
Esam1	endothelial cell-selective adhesion molecule	8
Fgd5	FYVE, RhoGEF and PH domain containing 5	0
Gpr116	G protein-coupled receptor 116	0
Grrp1	glycine/arginine rich protein 1	0
Hspa12b	Heat shock 70 kDa protein 12B	2
Icam1	Intercellular adhesion molecule-1 precursor	6104
Icam2	Intercellular adhesion molecule-2 precursor	87
Kdr	Vascular endothelial growth factor receptor 2 precursor	1398
Kifc1	Kinesin-like protein KIFC1 (Kinesin-like protein 2) (Kinesin-related protein HSET)	0
Lats2	large tumor suppressor 2	0
Lrrk1	leucine-rich repeat kinase 1	0
Mmrn2	elastin microfibril interfacer 3	0
Myo1b	Myosin Ib	0
NM_013753	paladin	0
NM_023516	RIKEN cDNA 2310016C08 gene	0
NM_153513	cDNA sequence BC028528	0
Notch4	Neurogenic locus notch homolog protein 4 precursor	66
Npnt	nephronectin	0
Npr3	Atrial natriuretic peptide clearance receptor precursor	0
Nrp	Neuropilin-1 precursor	101
Pecam	Platelet endothelial cell adhesion molecule precursor	829
Pltp	Phospholipid transfer protein precursor	17
Ptprb	protein tyrosine phosphatase, receptor type, B; vascular endothelial protein tyrosine phosphatase	3
Ptprm	Receptor-type protein-tyrosine phosphatase mu precursor	4
Ramp2	Receptor activity-modifying protein 2 precursor	31
Rasip1	Ras interacting protein 1	1
Robo4	Roundabout homolog 4 precursor	16
Sdpr	serum deprivation response	0
Slc43a3	solute carrier family 43, member 3; selectively expressed in embryonic epithelia protein-1	0
Slc9a3r2	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2	0
Slco2a1	Solute carrier organic anion transporter family member 2A1	2
Smad6	Mothers against decapentaplegic homolog 6	21
Sox13	SRY-box containing gene 13	0
Sox7	Transcription factor SOX-7	7
Stard9	START domain containing 9	0
Tenc1	C1 domain-containing phosphatase and tensin-like protein; tensin 2	0
Tie1	Tyrosine-protein kinase receptor Tie-1 precursor	74

Table 1. The 58-gene endothelial cluster. The "PubMed" column indicates the number of cocitations with the term "vascular" in PubMed abstracts at the timing of writing of this thesis.

number of transcripts that had not been previously linked to vascular development and function. At the time of writing of this thesis, 26 genes had no association with vascular biology in the published literature (Table 1).

3.4 The EC marker *DRAM* is associated with hypertension (Paper IV)

3.4.1 GWA studies and pathway analysis

Novel high-throughput technologies have enabled simultaneous genotyping of over one million genetic markers in a single experiment. Initial results from the first genome-wide association (GWA) studies revealed a large number of novel disease-

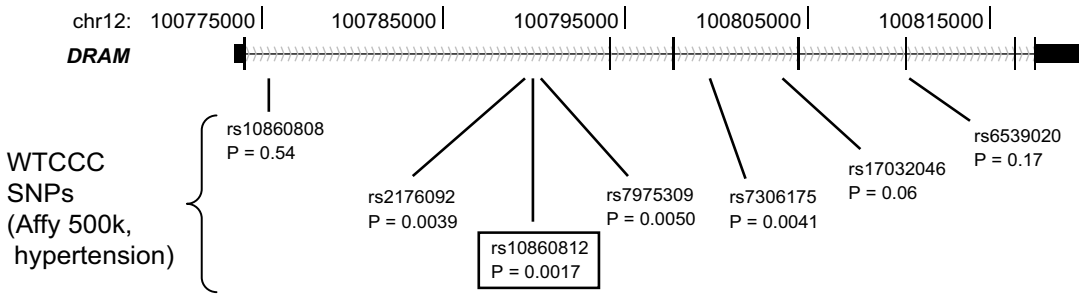


Figure 11. Mapping of GWA data to genes. Each gene was assigned association scores for each diseas. These scores were derived by identifying the strongest association signals in each gene locus.

associated single nucleotide polymorphisms (SNP) for several of the common complex diseases [160-165]. However, it has also become clear that both the individual and combined effects of those variations are small, and together they generally explain only a fraction of the total estimated heritability [166]. GWA studies published so far have reported only the most significant SNPs, with P-values low enough to be significant after correction for multiple testing, while ignoring the rest. Although this has led to the discovery of novel associations for complex diseases, a significant fraction of the heritable component is likely to be hidden among less significant SNPs that confer small disease risks. Based on the idea that polygenic diseases arise from the combined action of multiple genes within common pathways, *pathway analysis* has been suggested as a means to uncover more of the heritability from existing GWA data [167, 168]. Instead of focusing on individual SNPs, this approach is aimed at identifying functionally coupled gene sets, such as established pathways, in which disease-associated genes are statistically enriched.

3.4.2 Hypertension SNPs in endothelial genes

The endothelial marker genes identified in **Paper III** represents a high quality description of specific expression in the endothelium and includes genes that are functionally related in the sense that they have vital functions in the vasculature. We sought to investigate if there were diseases for which associated SNPs were overrepresented in this gene set, with the aim of discovering novel vascular-related susceptibility genes. The recently published (2007) WTCCC GWA study describes association statistics for ~500.000 SNPs derived using ~3000 common controls and ~2000 individuals for each of seven major diseases [161]. The study reported significant association signals ($P < 5e\text{-}7$) for coronary artery disease (CAD), type 1 diabetes (T1D), type 2 diabetes (T2D), rheumatoid arthritis (RA), Crohn's disease (CD) and bipolar disease (BD), but failed to identify significant loci for hypertension (HT). We assigned an association score to each gene (n = 14431),

Putative endothelial markers identified in **Paper III**:

Gene	Chr	Position	BD	CAD	CD	HT	RA	T1D	T2D
ADCY4	14	23857400	3.6E-01	2.5E-01	1.5E-03	4.1E-04	2.1E-03	3.0E-01	4.4E-02
GPR116	6	46928300	1.9E-02	1.1E-02	3.1E-03	5.4E-04	4.1E-05	3.9E-03	1.2E-02
FGD5	3	14835800	3.8E-02	1.6E-03	2.3E-02	8.1E-04	1.3E-02	1.0E-03	5.7E-03
NP_071926	5	141013000	2.3E-02	1.3E-02	6.8E-02	1.0E-03	2.3E-01	4.9E-02	6.3E-02
ITGA3	17	45488700	8.9E-03	1.7E-01	9.6E-02	1.5E-03	1.5E-01	3.0E-02	4.5E-02
DRAM	12	100774000	2.2E-01	4.2E-02	8.3E-02	1.8E-03	2.3E-02	2.2E-01	4.8E-02
ERG	21	38675700	7.4E-04	6.7E-03	2.5E-02	2.0E-03	2.4E-02	8.8E-03	7.0E-03
PTPRB	12	69201200	1.3E-02	8.7E-03	2.8E-03	3.5E-03	3.1E-02	1.3E-02	3.5E-02
EHD4	15	39978900	2.0E-01	3.2E-03	1.0E-01	3.7E-03	7.5E-02	2.4E-01	7.7E-02
SLCO2A1	3	135134000	6.3E-02	5.5E-02	8.6E-03	7.7E-03	2.5E-02	1.6E-01	3.2E-02
…	…	…	…	…	…	…	…	…	…

71 genes

P = 0.03 P = 8.2e-3

Figure 12. Enrichment of hypertension-associated SNPs among endothelial genes. The analysis was based on a list of 71 genes that were identified in Paper III.

derived by identifying the most significant SNP from the WTCCC study in each gene locus (Figure 11). This was done separately for all seven diseases.

The EC genes identified in **Paper III** (71 gene list) were evaluated for enrichment of disease-associated SNPs, using the same statistical method that was described in **Paper I** (Section 3.1.2) for the identification of regulatory motifs. Genes were tagged as associated/not associated using a threshold far below what was required for genome-wide significance in the WTCCC study ($P < 0.005$). The analysis revealed an enrichment of hypertension-associated genes among EC markers (Figure 12). Although borderline significant ($P = 0.057$, Bonferroni corrected with $n = 7$), we decided to test the hypothesis that genetic variation in genes expressed specifically in the endothelium may have a role in development of hypertension. The strongest SNPs in the top six genes (Figure 12) were selected for replication in an independent patient material. In addition we chose to include a SNP in the *NEBL* gene, due to its relatively strong association in the WTCCC study (P = 1.5e-5) and because *NEBL* has an expression pattern which nearly qualified it for inclusion in the 71-gene list. By purifying CD31+ microvascular fragments from mouse tissues using antibody-coated magnetic Dynabeads, we could confirm strong differential expression in microvessels for all selected genes using qRT-PCR.

3.4.3 Linking *DRAM* to hypertension and blood pressure

An independent evaluation of the selected SNPs was performed in 5208 hypertensive patients and 5297 population based controls. DNA for the case group was obtained from the NORDIL study, which included subjects with severe hypertension [169], while control DNA was derived from the MDC-CC study, which is a population based cohort [170]. One SNP (rs10860812) in the *DRAM*

gene was found to be robustly associated with hypertension diagnosis (P = 0.0078). This finding is still significant at the P < 0.05 level after Bonferroni correction. Exclusion of patients in the control group that were on antihypertensive treatment (n = 888) further increased the statistical significance. The minor allele (A) of rs10860812 was found to have a protective effect (OR = 0.93; 95% CI 0.88-0.98 per A-allele) and the direction was the same as in the WTCCC study.

Previous efforts to associate genetic variation in the population with hypertension have met with limited success [171, 172]. The first GWA studies for HT failed to identify novel gene associations, although a follow up of the top 6 SNPs from WTCCC HT study later revealed one association to blood pressure but not with hypertension diagnosis [173]. Recently, a GWA screen for hypertension in Amish subjects identified one novel association in the *STK39* gene [174]. rs10860812 is located in intron 1 of *DRAM*, and is not likely to be a direct disease-causing variant. The *DRAM* gene, which we concluded to be strongly enriched in the microvasculature of the kidney and the brain, is a regulator of autophagy that plays a critical role in apoptosis [175]. Apoptosis has been suggested to be of importance in hypertension-related vascular remodeling [176] and a central role for the kidney microvasculature in regulation of blood pressure is likewise firmly established [177]. *DRAM* is therefore a good candidate for a causal gene in this case. However, it should be noted that a number of other genes show various degree of linkage disequilibrium (LD) with this locus (the closest being *GNPTAB* and *CCDC53*), and further fine-mapping will be required to identify the causal SNP. Like most genetic variations that have been associated with common complex diseases, the effect size of the identified SNP was relatively small. However, the population frequency is high (0.46 for the protective A-allele), increasing its total contribution to disease in the population (population attributable risk, PAR).

To summarize, we could show that a genetic variant located in the DRAM gene, a regulator of apoptosis with strong differential expression in the microvasculature, is associated with development of hypertension in the human population.

3.5 Identification of microvascular miRNAs: role of miR-145 (Paper V)

During recent years, it has become clear that miRNAs have important roles in a wide range of biological processes. It is therefore natural that researchers have started to investigate the function of these molecules in vascular development and disease. Several studies have established a role for the miRNA pathway in angiogenesis and vasculogenesis. Knockout of the Dicer enzyme in mice leads to embryonic lethality and impaired angiogenesis and yolk sac formation [178] and siRNA knockdown of Dicer or Drosha leads to reduced endothelial proliferation, sprouting and network formation *in vitro* [179, 180]. The function of a number of

individual miRNAs in the vasculature has also been established, among them miR-126 which has strong endothelial expression and has been shown to control VCAM-1 expression in endothelial cells [181] and to regulate vascular integrity and angiogenesis *in vivo* in mouse and zebrafish [182-184]. However, it is likely that more miRNAs with important roles in the microvasculature remain to be identified. Several attempts have been made to apply microarray technology to identify miRNAs with strong or differential expression in endothelial cells. These have all been performed *in vitro* on HUVEC cells [179-181, 185] or on embryoid body (EB) cultures [183].

3.5.1 miR-145 is expressed in pericytes of the microvasculature

Specific/selective expression is a strong indicator of important function and we therefore screened publicly available datasets for miRNAs that appeared to be enriched in the mature microvasculature. A similar approach as the one described in **Paper III** was applied; six miRNAs were selected for further validation based on differential expression in two different highly vascularized structures; the lung and the kidney glomerulus. qRT-PCR on CD31+ vascular fragments isolated from four different adult mouse tissues revealed varying degrees of microvascular enrichment for all six miRNAs. In particular miR-126, a known endothelial marker, was consistently and strongly enriched in microvessels, followed by miR-145, which also showed convincing enrichment in fragments from all tissues. Microvascular expression of miR-145 has not been described, but one study found it to be highly expressed in the carotid artery [186] and it was recently shown to be expressed in mesangial cells of the kidney glomerulus and in smooth muscle [187]. These results suggested that it could be expressed in pericytes rather than endothelial cells. By investigating expression of miR-145 in vascular fragments from pericyte-deficient *Pdgfb*$^{ret/ret}$ mice and using *in situ* hybridization on mouse tissue sections we could establish that miR-145 is, in the microvasculature, expressed in pericytes.

3.5.2 miR-145 regulates *Fli1*

Since nothing was published on the molecular function of miR-145, we used target prediction software to identify possible functions for this miRNA in the vasculature. Interestingly, *Fli1*, an EC-expressed ETS family transcription factor that plays a crucial role in vascular development [188, 189] was highly ranked using several different algorithms. Using TargetScan [190] we could identify four possible miR-145 binding sites in the *Fli1* 3' UTR that were also evolutionarily conserved (Figure 13A). To evaluate if the predicted sites could mediate silencing by miR-145, these were cloned into the 3' UTRs of a series of luciferase reporter constructs. Cotransfection of these constructs with a miR-145 mimic dsRNA (Pre-miR-145) caused a significant reduction in reporter signal for all sites, while single base pair mutations in those sites reduced or abolished silencing (Figure 13B). Furthermore,

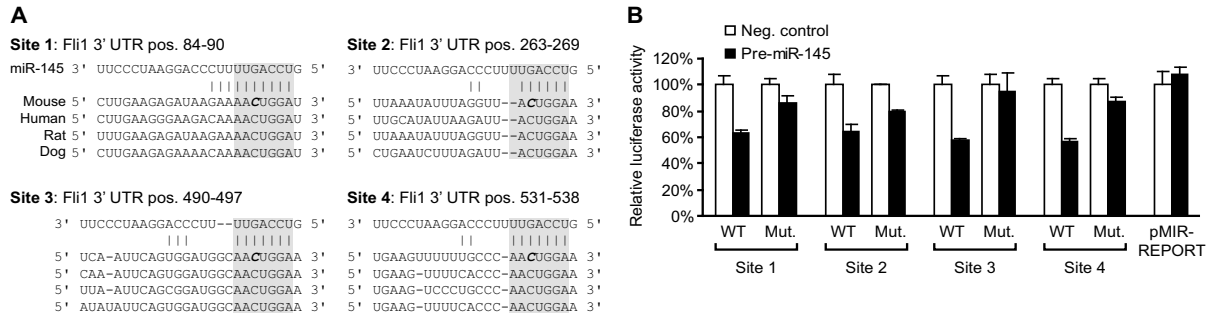


Figure 13. miR-145 regulates *Fli1*. (A) Four evolutionarily conserved miR-145 binding sites are present in the *Fli1* 3'UTR. Seed regions are indicated in grey. (B) Luciferase assays showing that all predicted sites can mediate silencing by miR-145. 60 bp regions containing wild-type (WT) and point mutated (Mut.) sites were cloned into a CMV-luciferase reporter and cotransfections with synthetic miR-145 or negative control were done in HEK/293 cells. Point mutations are indicated in bold/italic in (A).

western blot analysis showed that overexpression of miR-145 in mouse vascular aortic endothelial cells (VAEC) lead to downregulation of the FLI1 protein.

3.5.3 Modulation of cell migration by miR-145

Considering our results showing that microvascular expression of miR-145 is primarily due to expression in pericytes, the identification of the endothelial transcription factor FLI1 as a target of miR-145 was surprising. However, expression of miR-145 in the endothelium under certain conditions, or in low levels, is still possible. To study phenotypic effects of miR-145 overexpression, a synthetic miR-145 dsRNA was first introduced into VAEC cells cultured in growth factor supplemented medium. Using a wound healing assay, in which scratches were generated in a confluent cell layer and the amount of wound closure was determined 24 hours later, we could conclude that the migratory capability was reduced in cell transfected with synthetic miR-145. BrdU incorporation assays showed that cell proliferation was unchanged. Blood vessel growth *in vivo* is guided by growth factor gradients, and we therefore investigated cell migration in response to a gradient of VEGFA in a microfluidic migration chamber. Migration in the direction of the gradient was reduced by more than 50% in cells transfected with synthetic miR-145, while migration perpendicular to the gradient was unchanged. These findings suggest a role for miR-145 in regulation of cell migration in the vasculature. However, the results leave several questions unanswered. One of them is the role of miR-145 *in vivo*. Our preliminary results from overexpression of miR-145 in the developing zebrafish embryo did not result in a detectable phenotype; however, this could be due to technical issues. Another major unanswered question is the role of FLI1 in the observed phenotypic effects. FLI1 is an early marker of hemangioblast differentiation that has an important role in blood/vascular development and angiogenesis [189, 191-196]. It has also been shown to regulate

endoglin [197]. Endoglin is upregulated on the surface of proliferating and activated endothelial cells [198] and functions as an accessory receptor for the transforming growth factor β (TGB-β) family of proteins [199]. FLI1 is a potential mediator of the observed effects, but further studies, preferably rescue experiments, are needed to determine this. Finally, there is a discrepancy between the expression patterns of FLI1 and miR-145. One possibility is that FLI1, although considered to be an endothelial marker, is also expressed in pericytes under certain conditions (similar observations have been made for e.g. Ephrin-B2 [200] and VEGFR2/KDR [201]).

In conclusion, we identified miR-145 as a novel miRNA marker for pericytes. In addition, five other miRNAs with specific or selective expression in the microvascular endothelium were identified. Finally, we could show that miR-145 is a regulator of the *Fli1* gene and that overexpression of miR-145 leads to reduced cell migration *in vitro*.

4 Concluding remarks and future perspectives

This thesis presents findings in terms of novel regulators of gene expression, both in the vasculature (**Paper II, V**) and in other cells (**Paper I**). It also expands the current catalogue of vascular-specific marker genes, both protein-coding (**Paper III**) and non-coding (**Paper V**). In addition, it proposes that genetic variation in one of these newly identified vascular markers contributes to development of hypertension (**Paper IV**).

In **Paper I** we could confirm, using an unbiased computational approach, that there is modularity in the regulation of mammalian genes, in the sense that genes with similar expression patterns are often associated with common transcriptional regulators. Despite limitations in the amount of sequence that was analyzed and the number of transcription factors that were considered, we could rediscover several established regulatory mechanisms. Further analysis using the methods developed in this work suggested the zink finger transcription factor Zfp148 to be a putative regulator of ECM genes and EC-expressed genes, and the latter was later confirmed in zebrafish [202]. We have generated Zfp148-deficient mice but have yet to show the validity of these hypotheses *in vivo*. Several other predicted regulators remain to be validated.

LPP has been shown to play a role in SMC migration; an important mechanisms in vascular disease. This motivates studies on the regulation of LPP, and our conclusion that LPP is regulated by SRF/myocardin (**Paper II**) is a first step in this direction. However, a lot more remains to be done. The CArG box that mediates transcriptional activation of LPP (CArG 8) is located in a region that displays extremely high sequence conservation across a range of species, and a number of putative binding sites are present in this region (Figure 14). Interestingly, a PEA3 site is present in close proximity to the CArG box. It was recently shown that LPP can act as a transcriptional coactivator in complex with the ETS factor PEA3 [133], and this suggests a possible autoregulatory loop.

Many of the vital signaling molecules that control blood vessel development are expressed specifically by vascular cells, and in **Paper III** the number of known EC-specific/selective genes was essentially doubled. It seems reasonable that many of these novel markers have vital roles in the vasculature. Precise understanding of their function during vascular growth will require extensive experimental efforts using e.g. genetically engineered mice. Among the newly identified markers is *Gpr116* (Ig-hepta), an orphan receptor of the adhesion GPCR family, for which endothelium-selective expression has not been recognized [203]. Interestingly, a patent application was recently filed for a GPR116 agonist (USPTO 20080312281, OSI Pharmaceuticals). The inventors claim a possible use in treatment of obesity and diabetes, due to an observed expression in the pancreas, small instestine, colon

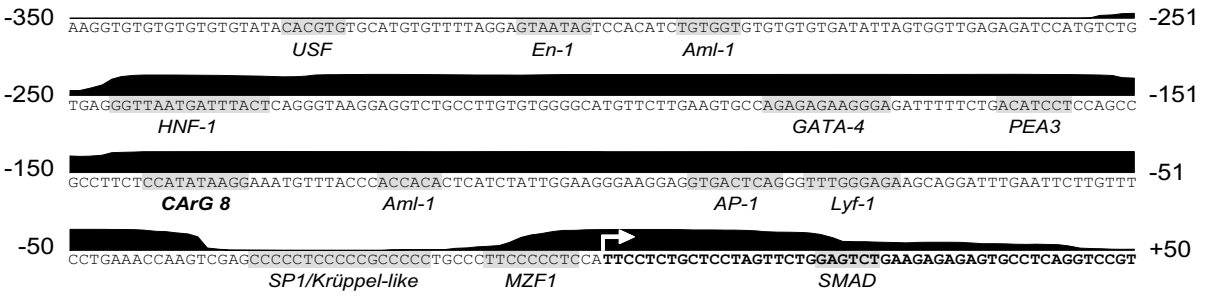


Figure 14. Putative regulatory elements in the intronic promoter of *LPP*. The black graph shows the degree of evolutionary conservation in a region covering -350...+50 relative the alternative transcription start in *LPP*. Putative binding sites from the TRANSFAC database are indicated.

and adipose tissue. Evaluation of the effect of this substance on angiogenic sprouting would be an exciting future prospect.

In **Paper IV**, we could show that a genetic variant located within in one of the newly identified EC markers, *DRAM*, was associated with development of human hypertension. However, the mechanism behind this association remains unclear, and fine mapping of the *DRAM* locus will be required to identify the causal variant. If *DRAM* is in fact involved in development of this disease – which is reasonable considering its expression pattern and putative role in vascular remodeling – this warrants further investigation of the underlying molecular mechanisms. No mouse knockout studies of *DRAM* have been published at the time of writing of this thesis, and this would be a natural continuation.

In **Paper V**, we identified miR-145 as a marker for pericytes in microvessels. Overexpression of miR-145 had an effect on cell migration *in vitro*, but this only represents a small initial step towards understanding of its function in the microvasculature. Although we identified the *Fli1* gene as a target for miR-145, the putative role of *Fli1* as a mediator of the migration effect remains to be determined. The list of predicted targets include other genes of interest in this context, e.g. vasorin (*Vasn*), which is expressed is VSMC and is involved in TGF-β signaling [204]. miR-145 is part of a polycistronic transcript that also contains miR-143, and these miRNAs are therefore coregulated. Consequently, miR-143 and its targets (which includes angiomotin, a protein that regulates endothelial cell migration and tube formation [205]) should be looked at. No knockout studies on mir-145/143 have been published. However, our attempts at overexpressing miR-145 in zebrafish did not result in a detectable phenotype. In addition to miR-145, a number of other miRNAs, including miR-23a, were identified as enriched in microvessels. The function of these molecules in the vasculature remains to be investigated.

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6 Additional publications

I. Transcriptional profiling reveals a critical role for tyrosine phosphatase VE-PTP in regulation of VEGFR2 activity and endothelial cell morphogenesis
Mellberg S, Dimberg A, Bahram F, Hayashi M, Rennel E, Ameur A, Westholm JO, Larsson E, Lindahl P, Cross MJ and Claesson-Welsh L
FASEB J, 2009. In press

II. Do two mutually exclusive gene modules define the phenotypic diversity of mammalian smooth muscle?
Larsson E, McLean SE, Mecham RP, Lindahl P and Nelander S.
Mol Genet Genomics, 2008. 280(2):127-37

III. HeliCis: a DNA motif discovery tool for colocalized motif pairs with periodic spacing
Larsson E, Lindahl P and Mostad P
BMC Bioinformatics, 2007. 28;8:418

IV. RhoA-dependent vascular smooth muscle cell-specific transcription: adding diaphanous formins to the puzzle
Larsson E, Zhou X and Akyürek LM
Arterioscler Thromb Vasc Biol, 2007. 27(3):448-9

V. New insights to vascular smooth muscle cell and pericyte differentiation of mouse embryonic stem cells in vitro
Lindskog H, Athley E, Larsson E, Lundin S, Hellström M and Lindahl P
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