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LYMPHANGIOGENOMICS

Genome-Wide Discovery and Functional Analysis of Novel Genes in Lymphangiogenesis

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Lymphangiogenomics is an Integrated Project of the European Commission's Sixth Framework Programme for Life Sciences, Genomics, and Biotechnology for Health (LSHG-CT-2004-503573) with 14 participating consortium members and 2 no longer active members (nr 7 and 12). Its aim was to thoroughly dissect the processes of lymphangiogenesis and to compare them with angiogenesis at the genetic, molecular, cellular, and functional level.

Project execution

The summary description of project objectives

The aim of this project was to discover novel genes important for lymphatic vascular versus blood vascular development and function and to study the functional role and therapeutic potential of their gene products in lymphangiogenesis using state-of-the-art technologies.

The lymphatic vasculature is essential for the maintenance of fluid balance in the body, for immune defence, and for the uptake of dietary fat. Absent or damaged lymphatic vessels may lead to lymphedema, a chronic and disfiguring swelling of the extremities, sometimes necessitating the amputation of the affected limb.

In addition, lymphatic vessels promote metastatic spread of cancer cells to distant organs - a leading cause of death in patients with cancer, and a major obstacle in the design of effective therapies. Lymphatic vessels were identified hundreds of years ago, yet very limited understanding exists of their development, function, and the molecular mechanisms underlying their disease process.

The methods we used included large-scale knockout and knock-down of the mouse genome, the embryonic stem (ES) cell technology, knock-down of zebra fish genes by morpholino-antisense and positional cloning of disease susceptibility genes involved in lymphangiogenesis.

Main objectives

This integrated project proposal aimed at thoroughly dissecting the processes of lymphangiogenesis and comparing this with angiogenesis at the genetic, molecular, cellular and functional level, with a strong emphasis on the discovery and analysis of novel key regulator genes of these vascular processes and the structures generated. Such study provided a critical basis for achieving a clear insight in and understanding of the biology and pathophysiology of the lymphatic system and its relationship with the blood vascular system. This knowledge is essential for further defining and evaluating new diagnostic approaches and therapeutic strategies to remedy lymphangiogenesis-related diseases. The proposal integrated various powerful genetic screening procedures, biotechnological tools and experimental analysis procedures, ranging from *in vitro* animal and human cell culture systems to *in vivo* animal models. The consortium combined highly competent, complementary and multidisciplinary participating research teams in this joint effort focused on the discovery and functional analysis of novel lymph/angiogenesis genes.

Specific scientific objectives were

(i) to *identify new genes and modifying factors* important for lymphatic vascular development and function. This was achieved using:

- several complementary gene-profiling set-ups (micro-array analysis) of lymphatic endothelial cells (LECs) versus blood vascular endothelial cells (BECs)
- micro-array analysis of diseased (tumour, malformations, etc) versus healthy and wild type versus gene-deficient LECs, isolated from animal models or from human patient samples
- quantitative Trait Locus (QTL) analysis of mouse strains resistant to versus sensitive for lymphatic vessel formation to identify modifier genes influencing lymphangiogenesis
- morpholino oligomer knockdown of related gene expression in zebra fish
- positional cloning of candidate disease genes and their evaluation in samples of lymph/vascular disorders

(ii) to characterize novel and known genes and determine their *functional role and importance* in lymphangiogenesis.

- depending on the timing, the role of the novel genes identified by the studies listed under (i) were further characterized in *in vitro* models and *in vivo* in mouse and zebra fish, using gene transfer or transfection
- RNA interference morpholino-knock-down in cells
- overexpression (zebra fish), and transgenic overexpression or gene targeting in mice
- protein products of some of these genes were evaluated by bioinformatics analysis of the LEC proteome
- the role of known genes implicated in lymphangiogenesis was further analyzed. These include LEC and/or blood endothelial and progenitor-specific genes such as VEGFR-3, VEGF-C, FOXC2, NP-2, podoplanin, Sox18, VE-cadherin, etc.
- their pathophysiological role was studied *in vitro* in cell lines derived from gene deficient mice, and *in vivo* in mice with single or combined gene deficiencies using experimental mouse models of physiological and pathological vascular and lymphatic vessel formation

(iii) to dissect specific *cellular mechanisms* of lymphangiogenesis and angiogenesis:

- mechanisms of angiogenic and lymphangiogenic sprouting
- lymphatic metastasis and invasion (*in vitro* tumour cell transmigration models; *in vivo* tumour lymphangiogenesis models)
- commitment of stem and progenitor cells towards LEC differentiation
- evaluation of the potential of progenitor cells for therapy
- proteomic and phage display evaluation of LEC signal transduction molecules and cell surface targets

Expected achievements

These studies provide a new and fundamental understanding of the molecular and cellular basis of lymphangiogenesis and therefore enable scientists to develop therapies to suppress the growth of lymphatic vessels (e.g. for cancer, inflammatory diseases) or to stimulate their growth (e.g. for tissue ischemia, lymphedema). The Integrated Project “Lymphangiogenomics” put forward ambitious, competitive research objectives addressing biological processes of high medical importance using a multidisciplinary analysis and validation approach.

Summary of main results and impact

Discovery of an essential role for Dll4/Notch signalling in the regulation of angiogenic sprouting

In sprouting angiogenesis, specialized endothelial tip cells lead the outgrowth of blood-vessel sprouts towards gradients of vascular endothelial growth factor (VEGF)-A. VEGF-A is also essential for the induction of endothelial tip cells, but it is not known how single tip cells are selected to lead each vessel sprout, and how tip-cell numbers are determined. We present evidence that delta-like 4 (Dll4)-Notch1 signalling regulates the formation of appropriate numbers of tip cells to control vessel sprouting and branching in the mouse retina. We show that inhibition of Notch signalling using gamma-secretase inhibitors, genetic inactivation of one allele of the endothelial Notch ligand Dll4, or endothelial-specific genetic deletion of Notch1, all promote increased numbers of tip cells. Conversely, activation of Notch by a soluble jagged1 peptide leads to fewer tip cells and vessel branches. Dll4 and reporters of Notch signalling are distributed in a mosaic pattern among endothelial cells of actively sprouting retinal vessels. At this location, Notch1-deleted endothelial cells preferentially assume tip-cell characteristics. Together, our results suggest that Dll4-Notch1 signalling between the endothelial cells within the angiogenic sprout serves to restrict tip-cell formation in response to VEGF, thereby establishing the adequate ratio between tip and stalk cells required for correct sprouting and branching patterns. This model offers an explanation for the dose-dependency and haploinsufficiency of the Dll4 gene, and indicates that modulators of Dll4 or Notch signalling, such as gamma-secretase inhibitors developed for Alzheimer's disease, might find usage as pharmacological regulators of angiogenesis.

Hellström, M. et al. *Nature* 445:776-780, 2007.

Discovery of a critical function for VEGFR3 in angiogenic sprouting

Angiogenesis, the growth of new blood vessels from pre-existing vasculature, is a key process in several pathological conditions, including tumour growth and age-related macular degeneration. Vascular endothelial growth factors (VEGFs) stimulate angiogenesis and lymphangiogenesis by activating VEGF receptor (VEGFR) tyrosine kinases in endothelial cells. VEGFR-3 (also known as FLT-4) is present in all endothelia during development, and in the adult it becomes restricted to the lymphatic endothelium. However, VEGFR-3 is upregulated in the microvasculature of tumours and wounds. We show that VEGFR-3 is highly expressed in angiogenic sprouts, and genetic targeting of VEGFR-3 or blocking of VEGFR-3 signalling with monoclonal antibodies results in decreased sprouting, vascular density, vessel branching and endothelial cell proliferation in mouse angiogenesis models. Stimulation of VEGFR-3 augmented VEGF-induced angiogenesis and sustained angiogenesis even in the presence of VEGFR-2 (also known as KDR or FLK-1) inhibitors, whereas antibodies against VEGFR-3 and VEGFR-2 in combination resulted in additive inhibition of angiogenesis and tumour growth. Furthermore, genetic or pharmacological disruption of the Notch signalling pathway led to widespread endothelial VEGFR-3 expression and excessive sprouting, which was inhibited by blocking VEGFR-3 signals. Our results implicate VEGFR-3 as a regulator of vascular network formation. Targeting VEGFR-3 may provide additional efficacy for anti-angiogenic therapies, especially towards vessels that are resistant to VEGF or VEGFR-2 inhibitors.

Tammela, T. et al. *Nature* 454:656-660, 2008.

Identification of the transcriptome of highly permeable vessels in the kidney glomerulus and the role for Foxc2 in glomerulus development

To advance our understanding of development, function and diseases in the kidney glomerulus, we have established and large-scale sequenced cDNA libraries from mouse glomeruli at different stages of development, resulting in a catalogue of 6053 different genes. The glomerular cDNA clones were arrayed and hybridized against a series of labelled targets from isolated glomeruli, non-glomerular kidney tissue, FACS-sorted podocytes and brain capillaries, which identified over 300 glomerular cell-enriched transcripts, some of which were further sublocalized to podocytes, mesangial cells and juxtaglomerular cells by *in situ* hybridization. For the earliest podocyte marker identified, Foxc2, knockout mice were used to analyze the role of this protein during glomerular development. We show that Foxc2 controls the expression of a distinct set of podocyte genes involved in podocyte differentiation and glomerular basement membrane maturation. The primary podocyte defects also cause abnormal differentiation and organization of the glomerular vascular cells. We surmise that studies on the other novel glomerulus-enriched transcripts identified in this study will provide new insight into glomerular development and pathomechanisms of disease.

Takemoto, M. et al. *EMBO J*, 25:1160-1174, 2006.

Identification of a core set of 58 gene transcripts with broad and specific expression in the microvasculature

Pathological angiogenesis is an integral component of many diseases. Anti-angiogenesis and vascular targeting are therefore promising new therapeutic principles. However, few endothelial-specific putative drug targets have been identified, and information is still limited about endothelial-specific molecular processes. We aimed at determining the endothelial cell-specific core transcriptome *in vivo*. Analysis of publicly available microarray data identified a mixed vascular/lung cluster of 132 genes that correlated with known endothelial markers. Filtering against kidney glomerular/non-glomerular and brain vascular/nonvascular microarray profiles separated contaminating lung markers, leaving 58 genes with broad and specific microvascular expression. More than half of these have not previously been linked to endothelial functions or studied in detail before. The endothelial cell-specific expression of a selected subset of these, Eltd1, Gpr116, Ramp2, Slc9a3r2, Slc43a3, Rasip1, and NM_023516, was confirmed by real-time quantitative polymerase chain reaction and/or immunohistochemistry. We used a combination of publicly available and own microarray data to identify 58 gene transcripts with broad yet specific expression in microvascular endothelium. Most of these have unknown functions, but many of them are predicted to be cell surface expressed or implicated in cell signalling processes and should therefore be explored as putative microvascular drug targets.

Wallgard, E. et al. *Atherscler. Thomb. Vasc. Biol*. 28:1469-1476, 2008

Identification of pericytes as crucial players in lymph and blood borne metastasis

Previously we observed that neural cell adhesion molecule (NCAM) deficiency in beta tumour cells facilitates metastasis into distant organs and local lymph nodes. Here, we show that NCAM-deficient beta cell tumours grew leaky blood vessels with perturbed pericyte-endothelial cell-cell interactions and deficient perivascular deposition of ECM components. Conversely, tumour cell expression of NCAM in a fibrosarcoma model (T241) improved pericyte recruitment and increased perivascular deposition of ECM molecules. Together, these findings suggest that NCAM may limit tumour cell metastasis by stabilizing the microvessel wall. To directly address whether pericyte dysfunction increases the metastatic potential of solid tumours, we studied beta cell tumourigenesis in primary pericyte-deficient Pdgfb(ret/ret) mice. This resulted in beta tumour cell metastases in

distant organs and local lymph nodes, demonstrating a role for pericytes in limiting tumour cell metastasis. These data support a new model for how tumour cells trigger metastasis by perturbing pericyte-endothelial cell-cell interactions.

Xian, X. et al. *J. Clin. Invest.* 116:642-651, 2006.

Therapeutic differentiation and maturation of lymphatic vessels after lymph node dissection and transplantation

Surgery or radiation therapy of metastatic cancer often damages lymph nodes, leading to secondary lymphedema. We showed, using a newly established mouse model, that collecting lymphatic vessels can be regenerated and fused to lymph node transplants after lymph node removal. Treatment of lymph node-excised mice with adenovirally delivered vascular endothelial growth factor-C (VEGF-C) or VEGF-D induced robust growth of the lymphatic capillaries, which gradually underwent intrinsic remodelling, differentiation and maturation into functional collecting lymphatic vessels, including the formation of uniform endothelial cell-cell junctions and intraluminal valves. The vessels also reacquired pericyte contacts, which downregulated lymphatic capillary markers during vessel maturation. Growth factor therapy improved the outcome of lymph node transplantation, including functional reconstitution of the immunological barrier against tumour metastasis. These results show that growth factor-induced maturation of lymphatic vessels is possible in adult mice and provide a basis for future therapy of lymphedema.

Tammela T. et al. *Nat. Med.* 13:1458-66, 2007.

Vascular endothelial cell growth factor receptor 3-mediated activation of lymphatic endothelium is crucial for tumour cell entry and spread via lymphatic vessels

Lymphangiogenic growth factors vascular endothelial growth factor (VEGF)-C and VEGF-D have been shown to promote lymphatic metastasis by inducing tumour-associated lymphangiogenesis. We investigated how tumour cells gain access into lymphatic vessels and at what stage tumour cells initiate metastasis. We show that VEGF-C produced by tumour cells induced extensive lymphatic sprouting towards the tumour cells as well as dilation of the draining lymphatic vessels, suggesting an active role of lymphatic endothelial cells in lymphatic metastasis. A significant increase in lymphatic vessel growth occurred between 2 and 3 weeks after tumour xenotransplantation, and lymph node metastasis occurred at the same stage. These processes were blocked dose-dependently by inhibition of VEGFR-3 signalling by systemic delivery of a soluble VEGFR-3-immunoglobulin (Ig) fusion protein via adenoviral or adeno-associated viral vectors. However, VEGFR-3-Ig did not suppress lymph node metastasis when the treatment was started at a later stage after the tumour cells had already spread out, suggesting that tumour cell entry into lymphatic vessels is a key step during tumour dissemination via the lymphatics. Whereas lymphangiogenesis and lymph node metastasis were significantly inhibited by VEGFR-3-Ig, some tumour cells were still detected in the lymph nodes in some of the treated mice. This indicates that complete blockade of lymphatic metastasis may require the targeting of both tumour lymphangiogenesis and tumour cell invasion.

He Y. et al. *Cancer Res.* 65:4739-46, 2005.

Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis

Lymphatic vessels are essential for the removal of interstitial fluid and prevention of tissue oedema. Lymphatic capillaries lack associated mural cells, and collecting lymphatic vessels have valves,

which prevent lymph backflow. In lymphedema-distichiasis (LD), lymphatic vessel function fails because of mutations affecting the forkhead transcription factor FOXC2. We found that Foxc2(-/-) mice show abnormal lymphatic vascular patterning, increased pericyte investment of lymphatic vessels, agenesis of valves and lymphatic dysfunction. In addition, an abnormally large proportion of skin lymphatic vessels was covered with smooth muscle cells in individuals with LD and in mice heterozygous for Foxc2 and for the gene encoding lymphatic endothelial receptor, Vegfr3. Our data show that Foxc2 is essential for the morphogenesis of lymphatic valves and the establishment of a pericyte-free lymphatic capillary network and that it cooperates with Vegfr3 in the latter process. Our results indicate that an abnormal interaction between the lymphatic endothelial cells and pericytes, as well as valve defects, underlie the pathogenesis of LD.

Petrova, T. et al. *Nat. Med.* 10:974-81, 2004.

Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins

Lymphatic vessels are essential for immune surveillance, tissue fluid homeostasis and fat absorption. Defects in lymphatic vessel formation or function cause lymphedema. We have shown that the vascular endothelial growth factor C (VEGF-C) is required for the initial steps in lymphatic development. In Vegfc-/- mice, endothelial cells commit to the lymphatic lineage but do not sprout to form lymph vessels. Sprouting was rescued by VEGF-C and VEGF-D but not by VEGF, indicating VEGFR-3 specificity. The lack of lymphatic vessels resulted in prenatal death due to fluid accumulation in tissues, and Vegfc+/- mice developed coetaneous lymphatic hypoplasia and lymphedema. Our results indicate that VEGF-C is the paracrine factor essential for lymphangiogenesis, and show that both Vegfc alleles are required for normal lymphatic development.

Karkkainen, M.J. et al. *Nat. Immunol.* 5:74-80, 2004.

Neural guidance molecules regulate vascular remodelling and vessel navigation

The development of the embryonic blood vascular and lymphatic systems requires the coordinated action of several transcription factors and growth factors that target endothelial and periendothelial cells. However, according to recent studies, the precise "wiring" of the vascular system does not occur without an ordered series of guidance decisions involving several molecules initially discovered for axons in the nervous system, including ephrins, netrins, slits, and semaphorins. We can now summarize the new advances in our understanding of the roles of these axonal path finding molecules in vascular remodelling and vessel guidance, indicating that neuronal axons and vessel sprouts use common molecular mechanisms for navigation in the body.

Eichmann A. et al. *Genes & Dev.* 19:1013-1021, 2005.

Neural guidance genes contribute to blood and lymphatic vessel patterning

Blood vessels and nerves are structurally similar complex branched systems. Their guidance must be exquisitely regulated to ensure proper wiring of both networks. Recent results showed that specialized endothelial cells, resembling axonal growth cones, form the tips of growing capillaries. These endothelial tip cells guide outgrowing capillaries in response to gradients of extracellular matrix-bound vascular endothelial growth factor. Several axon guidance molecules, including Semaphorins, Netrins, Ephrins and Slits, have also been implicated in vessel path finding and

network formation. In particular, Semaphorin3E and its receptor plexinD1 in addition to the Netrin receptor UNC5B have recently been shown to direct endothelial tip cell navigation.

Eichmann A. et al. *Curr. Op. Neurobiol.* 15:108-115, 2005.

Neuropilin-2 mediates VEGF-C induced lymphatic sprouting together with VEGFR-3

Vascular sprouting is a key process driving development of the vascular system. Despite this, little is known about the molecular mediators that regulate this critical biology, particularly for lymphatic vessels. We showed that neuropilin-2 (Nrp2), a transmembrane receptor for class 3 semaphorins and VEGF family members, including the lymphangiogenic growth factor VEGF-C, plays an important role in sprouting. We demonstrate that blocking of VEGF-C binding to Nrp2 using a monoclonal antibody specifically blocks sprouting of developing lymphatic endothelial tip cells in vivo. Blocking VEGF-C binding to Nrp2 in vitro shows that Nrp2 plays a selective role in driving sprout formation by modulating lymphatic endothelial tip-cell extension and preventing tip cell stalling and retraction. Genetic deletion of Nrp2 reproduces the sprouting defects seen after antibody treatment. To investigate if this defect depends on Nrp2 interaction with VEGFR-2 and/or 3, we intercrossed heterozygous mice lacking one allele of these receptors. Double-heterozygous nrp2vegfr2 mice developed normally without detectable lymphatic sprouting defects. In contrast, double heterozygote nrp2vegfr3 mice showed a reduction of lymphatic vessel sprouting and reduced lymph vessel coverage in adult organs. Thus, interaction between Nrp2 and VEGFR-3 mediates proper lymphatic vessel sprouting in response to VEGF-C.

Xu, Y. et al. *J. Cell Biol*. in press.

Activation of the UNC5B receptor by Netrin-1 inhibits sprouting angiogenesis

Netrins are secreted molecules with roles in axonal growth and angiogenesis. The Netrin receptor UNC5B is required during embryonic development for vascular patterning, suggesting that it may also contribute to postnatal and pathological angiogenesis. We have shown that unc5b is down-regulated in quiescent adult vasculature, but re-expressed during sprouting angiogenesis in matrigel and tumour implants. Stimulation of UNC5B-expressing neovessels with an agonist (Netrin-1) inhibits sprouting angiogenesis. Genetic loss of function of unc5b reduces Netrin-1-mediated angiogenesis inhibition. Expression of UNC5B full-length receptor also triggers endothelial cell repulsion in response to Netrin-1 *in vitro*, whereas a truncated UNC5B lacking the intracellular signalling domain fails to induce repulsion. These data show that UNC5B activation inhibits sprouting angiogenesis, thus identifying UNC5B as a potential anti-angiogenic target.

Larrivée, B. et al. *Genes & Dev*. 21:2433-2447, 2007.

The Netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system

Blood vessels and nerves are complex, branched structures that share a high degree of anatomical similarity. Guidance of vessels and nerves has to be exquisitely regulated to ensure proper wiring of both systems. Several regulators of axon guidance have been identified and some of these are also expressed in endothelial cells; however, the extent to which their guidance functions are conserved in the vascular system is still incompletely understood. We showed that the repulsive netrin receptor UNC5B is expressed by endothelial tip cells of the vascular system. Disruption of the Unc5b gene in mice, or of Unc5b or netrin-1a in zebra fish, leads to aberrant extension of endothelial tip cell filopodia, excessive vessel branching and abnormal navigation. Netrin-1 causes

endothelial filopodial retraction, but only when UNC5B is present. Thus, UNC5B functions as a repulsive netrin receptor in endothelial cells controlling morphogenesis of the vascular system.

Lu, X. et al. *Nature* 432:179-186, 2004.

The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching

Delta-like 4 (Dll4) is a transmembrane ligand for Notch receptors that is expressed in arterial blood vessels and sprouting endothelial cells. We showed that Dll4 regulates vessel branching during development by inhibiting endothelial tip cell formation. Heterozygous deletion of dll4 or pharmacological inhibition of Notch signalling using gamma-secretase inhibitor revealed a striking vascular phenotype, with greatly increased numbers of filopodia-extending endothelial tip cells and increased expression of tip cell marker genes compared with controls. Filopodia extension in dll4(+/-) retinal vessels required the vascular growth factor VEGF and was inhibited when VEGF signalling was blocked. Although VEGF expression was not significantly altered in dll4(+/-) retinas, dll4(+/-) vessels showed increased expression of VEGFR-2 and decreased expression of VEGFR-1 compared with wild-type, suggesting they could be more responsive to VEGF stimulation. In addition, expression of dll4 in wild-type tip cells was itself decreased when VEGF signalling was blocked, indicating that dll4 may act downstream of VEGF as a "brake" on VEGF-mediated angiogenic sprouting. Taken together, these data reveal Dll4 as a negative regulator of vascular sprouting and vessel branching that is required for normal vascular network formation during development.

Suchting, S. et al. *Proc. Natl. Acad. Sci. USA* 104:3225-3230, 2007.

VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain

Vascular endothelial growth factor C (VEGF-C) was first identified as a regulator of the vascular system, where it is required for the development of lymphatic vessels. Here we report actions of VEGF-C in the central nervous system. We detected the expression of the VEGF-C receptor VEGFR-3 in neural progenitor cells in *Xenopus laevis* and mouse embryos. In *Xenopus* tadpole VEGF-C knockdowns and in mice lacking Vegfc, the proliferation of neural progenitors expressing VEGFR-3 was severely reduced, in the absence of intracerebral blood vessel defects. In addition, Vegfc-deficient mouse embryos showed a selective loss of oligodendrocyte precursor cells (OPCs) in the embryonic optic nerve. *In vitro*, VEGF-C stimulated the proliferation of OPCs expressing VEGFR-3 and nestin-positive ventricular neural cells. VEGF-C thus has a new, evolutionary conserved function as a growth factor selectively required by neural progenitor cells expressing its receptor VEGFR-3.

Le Bras, B. et al. *Nat. Neuroscience* 9:340-348, 2006.

Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates

Renal transplant rejection is caused by a lymphocyte-rich inflammatory infiltrate that attacks cortical tubules and endothelial cells. Immunosuppressive therapy reduces the number of infiltrating cells; however, their exit routes are not known. We found a >50-fold increase of lymphatic vessel density over normal kidneys in grafts with nodular mononuclear infiltrates is demonstrated by immunohistochemistry on human renal transplant biopsies using antibodies to the lymphatic endothelial marker protein podoplanin. Nodular infiltrates are constantly associated with newly

formed, Ki-67-expressing lymphatic vessels and contain the entire repertoire of T and B lymphocytes to provide specific cellular and humoral alloantigenic immune responses, including Ki-67(+) CD4(+) and CD8(+) T lymphocytes, S100(+) dendritic cells, and Ki-67(+)CD20(+) B lymphocytes and lambda- and kappa-chain-expressing plasmacytoid cells. Numerous chemokine receptor CCR7(+) cells within the nodular infiltrates seemed to be attracted by secondary lymphatic chemokine (SLC/CCL21) that is produced and released by lymphatic endothelial cells in a complex with podoplanin. From these results, it is speculated that lymphatic neoangiogenesis not only contributes to the export of the rejection infiltrate but also is involved in the maintenance of a potentially detrimental alloreactive immune response in renal transplants and provides a novel therapeutic target.

Kerjaschki, D. et al. *J. Am. Soc. Nephrol.* 15:603-12, 2004**.**

Lymphatic endothelial progenitor cells contribute to *de novo* lymphangiogenesis in human renal transplants

De novo lymphangiogenesis influences the course of different human diseases as diverse as chronic renal transplant rejection and tumour metastasis. The cellular mechanisms of lymphangiogenesis in human diseases are currently unknown, and could involve division of local pre-existing endothelial cells or incorporation of circulating progenitors. We analyzed renal tissues of individuals with gender-mismatched transplants who had transplant rejection and high rates of overall lymphatic endothelial proliferation as well as massive chronic inflammation. Donor-derived cells were detected by *in situ* hybridization of the Y chromosome. We compared these tissues with biopsies of essentially normal skin and intestine, and two rare carcinomas with low rates of lymphatic endothelial proliferation that were derived from individuals with gender-mismatched bone marrow transplants. Here, we provide evidence for the participation of recipient-derived lymphatic progenitor cells in renal transplants. In contrast, lymphatic vessels of normal tissues and those around post-transplant carcinomas did not incorporate donor-derived progenitors. This indicates a stepwise mechanism of inflammation-associated de novo lymphangiogenesis, implying that potential lymphatic progenitor cells derive from the circulation, transmigrate through the connective tissue stroma, presumably in the form of macrophages, and finally incorporate into the growing lymphatic vessel.

Kerjaschki, D. et al. *Nat. Med.* 12:230-4, 2006.

Podoplanin-induced platelet aggregation mediates separation of blood and lymphatic vessels

Separation of lymphatic from blood circulatory systems by a hitherto unknown mechanism is pivotal for lymphatic transport of protein rich fluids back from the tissues to the blood. Podoplanin is expressed specifically on lymphatic but not on blood endothelial cells and mediates platelet activation, leading us to speculate whether podoplanin dependent platelet activation might be responsible for separation of blood and lymphatic vessels. Podoplanin$^{-/-}$ mice developed a lymphatic network filled with blood between ~E13.5 and ~E16.5 that regressed thereafter. Platelet aggregates were found at the entrances of sprouting lymphatic sacs in wild type but not in podoplanin$^{-/-}$ embryos. Administration of a podoplanin inhibitory antibody to pregnant mice as well as of acetyl salicylic acid that inhibits platelet aggregation mimicked the “non-separation” phenotype seen in podoplanin$^{-/-}$ mice, as did mice deficient in kindlin-3, an adhesion plaque protein that is indispensable for platelet integrin activation. Similarly, during wound healing lymphatic vessels develop that were plugged by platelet aggregates in adult wild type but not in podoplanin$^{-/-}$ mice, and that were in turn filled with blood in the podoplanin deficient animals. From these data we conclude that podoplanin expressed specifically on developing lymphatic endothelium is

responsible for separation of blood from lymphatic vessels by inducing local platelet aggregation and in turn plugging of the orifices of developing lymphatic vessels.

Uhrin, P. et al. *Blood,* in press.

Lymphatic Pre-collectors Contain a Novel, Specialized Subpopulation of Podoplanin$_{low}$, CCL27-Expressing Lymphatic Endothelial Cells

Expression of the lymphoendothelial marker membrane mucoprotein podoplanin (podo) distinguishes endothelial cells of both blood and lymphatic lineages. We have discovered two distinct subpopulations of lymphatic endothelial cells (LECs) in human skin that were defined by their cell surface densities of podoplanin and were designated LEC podo-low and LEC podo-high. LEC podo-low is restricted to lymphatic pre-collector vessels that originate from initial LEC podo-high-containing lymphatic capillaries and selectively express several pro-inflammatory factors. In addition to the chemokine receptor protein Duffy blood group antigen receptor for chemokines, these factors include the constitutively expressed chemokine CCL27, which is responsible for the accumulation of pathogenic CCR10+ T lymphocytes in human inflammatory skin diseases. In this study, we report that CCR10+ T cells accumulate preferentially both around and within CCL27+ LEC podo-low pre-collector vessels in skin biopsies of human inflammatory disease. In transmigration assays, isolated CCR10+ T lymphocytes are chemotactically attracted by LEC podo-low in a CCL27-dependent fashion, but not by LEC podo-high. These observations indicate that LEC podo-low-containing pre-collector vessels constitute a specialized segment of the initial lymphatic microvasculature, and we hypothesize that these LEC podo-low-containing vessels are involved in the trafficking of CCR10+ T cells during skin inflammation.

Wick, N. et al. *Am. J. Pathol.* 173:1202-9, 2008.

Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism

In salt-sensitive hypertension, the accumulation of Na(+) in tissue has been presumed to be accompanied by a commensurate retention of water to maintain the isotonicity of body fluids. We showed that a high-salt diet (HSD) in rats leads to interstitial hypertonic Na(+) accumulation in skin, resulting in increased density and hyperplasia of the lymphcapillary network. The mechanisms underlying these effects on lymphatics involve activation of tonicity-responsive enhancer binding protein (TonEBP) in mononuclear phagocyte system (MPS) cells infiltrating the interstitium of the skin. TonEBP binds the promoter of the gene encoding vascular endothelial growth factor-C (VEGF-C, encoded by Vegfc) and causes VEGF-C secretion by macrophages. MPS cell depletion or VEGF-C trapping by soluble VEGFR-3 blocks VEGF-C signalling, augments interstitial hypertonic volume retention, decreases endothelial nitric oxide synthase expression and elevates blood pressure in response to HSD. Our data show that TonEBP-VEGF-C signalling in MPS cells is a major determinant of extracellular volume and blood pressure homeostasis and identify VEGFC as an osmosensitive, hypertonicity-driven gene intimately involved in salt-induced hypertension.

Machnik, A. et al. *Nat. Med.* 15:545-52, 2009.

Tumour invasion into intrametastatic lymphatics causes lymph node metastasis

In human mammary carcinomas the status of axillary lymph node metastasis is a prognostically relevant predictor of distant organ metastasis and clinical outcome. Most frequently metastasis formation starts in the axillary sentinel lymph node, and progresses *via* connecting lymphatic vessels into post-sentinel lymph nodes. However, the mechanisms of consecutive lymph node

colonization are unknown. We have analyzed the interplay of tumour cells and lymphatic vessels in 104 human mammary carcinomas including their matching axillary lymph nodes. Thereby we identified *de novo* formation of intrametastatic lymphatic vessels and bulk tumour cell invasion as critical steps in the formation of post-sentinel metastasis. Mechanistic insights obtained *in vitro* revealed a predominant role of tumour derived 15-lipoxygenase, and one of its arachidonic acid metabolites, 12(S)-hydroxyeicosatetraenoic acid in the invasion process. The importance of these mediators was confirmed in a xenograft tumour model in which shRNA-knock down of 15-lipoxygenase repressed lymph node metastasis.

Kerjaschki, D. et al. *Nature,* in revision.

Functionally specialized junctions between endothelial cells of lymphatic vessels

Intercellular junctions in initial lymphatic vessels are considered to function as sites for entry of fluid and cells into the lymphatics in inflammation. Despite this specialized function their structural organization is largely unknown. We have identified novel discontinuous button-like junctions, which were uniquely present in initial lymphatic vessels. Button-like junctions formed anchoring points at the bases of overlapping flaps of oak-leaf shaped endothelial cell borders, thus resembling valve-like structures. Similarly to continuous, zipper-like junctions in collecting lymphatics, these junctions were composed of both adherens and tight junction proteins. Interestingly, PECAM-1, known to promote leukocyte trafficking through junctions, was distributed at flaps in between the buttons. This suggested a role for PECAM-1 in migration of leukocytes through these valve-like structures and indeed, activated dendritic cells transmigrating into initial lymphatics at the level of such PECAM-1-enriched flaps were identified in inflamed mouse airways. Moreover, tip cells at the growing ends of lymphangiogenic sprouts in inflamed airways had continuous junctions. As these sprouts developed into new lymphatic vessels, however, button-like junctions were formed, suggesting that they represent features of differentiated, highly specialized endothelial cells. In summary, our results show that button-like junctions in initial lymphatic vessels support entry of fluid and cells without disrupting the overall integrity.

Baluk, P. et al. *J. Exp. Med*. Oct 1;204(10):2349-62, 2007.

Sox18 induces development of the lymphatic vasculature in mice

The lymphatic system plays a key role in tissue fluid regulation and tumour metastasis, but its origins in the embryo remain controversial. Lymphatic vessels are thought to arise from a population of endothelial precursor cells budding from the cardinal vein and expressing the lymphatic marker PROX1. Defects in *SOX18* cause lymphatic dysfunction in the human syndrome hypotrichosis-lymphedema-telangiectasia (HLT), suggesting a role for the SOX18 transcription factor in lymphatic development or function. We used molecular, cellular and *in vivo* genetic assays in mice to demonstrate a critical role for SOX18 in this system. SOX18 directly activates transcription from the *Prox1* promoter. Over-expression of SOX18 in mice induces ectopic formation of lymphatic endothelial cells, while *Sox18*-null embryos show a complete absence of PROX1-positive lymphatic endothelial cells emanating from the cardinal vein. Our findings place SOX18 at the head of the transcriptional hierarchy leading to development of lymphatic vessels.

François, M. et al. *Nature* 456(7222):643-7, 2008.

Sox18 and Sox7 play redundant roles in vascular development

Mutations in *SOX18* cause the human Hypotrichosis-Lymphedema-Telangiectasia (HLT) syndrome. Their murine counterparts are the spontaneous *ragged* mutants, showing combined

defects in hair follicle, blood vessel and lymphatic vessel development. Mice null for *Sox18* display only mild coat defects, suggesting a dominant negative effect of *Sox18/ragged* mutations and functional redundancy between Sox18 and other Sox-F proteins. We addressed this point in zebra fish. The zebra fish homologues of *Sox18* and of *Sox7* are expressed in angioblasts and in the endothelial component of nascent blood vessels in embryos. Knockdown of either gene, using moderate doses of specific morpholinos, had minimal effects on vessels. In contrast, simultaneous knockdown of both genes resulted in multiple fusions between the major axial vessels. With combined use of transgenic lines and molecular markers, we could show that endothelial cells are specified, but fail to acquire a correct arteriovenous identity. Venous endothelial cell differentiation was more severely affected than arterial. Thus, *sox7* and *sox18* play redundant but collectively essential roles in the establishment of proper arteriovenous identity in zebra fish. Our data suggest that a defect in arteriovenous identity could be responsible for the formation of telangiectases in HLT patients.

Cermenati, S. et al. *Blood* 111:2657-66, 2008.

Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5

Intercellular junctions mediate adhesion and communication between adjoining cells. Although formed by different molecules, tight junctions (TJs) and adherens junctions (AJs) are functionally and structurally linked but the signalling pathways behind this interaction are unknown. We have described a novel and cell-specific mechanism of cross talk between these two types of structures. We show that endothelial VE-cadherin at AJs up-regulates the TJ adhesive protein claudin-5. This effect requires the release of the inhibitory activity of forkhead box factor FoxO1 and Tcf-4-β-catenin transcriptional repressor complex. VE-cadherin acts by inducing FoxO1 phosphorylation through AKT activation and by limiting β-catenin nuclear translocation. These results offer a molecular basis for the link between AJ and TJ and explain why VE-cadherin inhibition may cause dramatic increase in permeability.

Taddei, A. et al. *Nat. Cell Biol*. 10(8):923-34, 2008.

VE-cadherin is a critical endothelial regulator of TGF-beta signalling

VE-cadherin is an endothelial specific transmembrane protein concentrated at cell-to-cell adherens junctions. Besides promoting cell adhesion and controlling vascular permeability, VE-cadherin transfers intracellular signals that contribute to vascular stabilization. However, the molecular mechanism by which VE-cadherin regulates vascular homeostasis is still poorly understood. We found that VE-cadherin expression and junctional clustering are required for optimal transforming growth factor-b (TGF-b) signalling in endothelial cells (ECs). TGF-b anti-proliferative and anti-migratory responses are increased in the presence of VE-cadherin. ECs lacking VE-cadherin are less responsive to TGF-b/ALK1- and TGF-b/ALK5-induced Smad phosphorylation and target gene transcription. VE-cadherin co-immunoprecipitates with all the components of the TGF-b receptor complex: TbRII, ALK1, ALK5 and endoglin. Data show that clustered VE-cadherin recruits TbRII and promotes TGF-b signalling by enhancing TbRII/TbRI assembly into an active receptor complex. Taken together our data indicate that VE-cadherin is a positive and EC specific regulator of TGF-b signalling. This suggests that reduction or inactivation of VE-cadherin may contribute to the pathological effects of impaired TGF-b signalling.

Rudini, N. et al. *EMBO J.* Apr 9;27(7):993-1004, 2008.

Tumour invasion in the absence of epithelial-mesenchymal transition: podoplanin-mediated remodelling of the cytoskeleton

The expression of podoplanin, a small mucin-like protein widely used as marker for lymphatic endothelial cells, is upregulated in the invasive front of a number of human carcinomas. We have investigated podoplanin function in cultured human breast cancer cells, in a mouse model of pancreatic β cell carcinogenesis, and in human cancer biopsies. Our results indicate that podoplanin promotes tumour cell invasion *in vitro* and *in vivo*. Notably, the expression and subcellular localization of epithelial markers are unaltered and mesenchymal markers are not induced in invasive podoplanin-expressing tumour cells. Rather, podoplanin induces collective cell migration by filopodia formation via the downregulation of the activities of small Rho family GTPases. In conclusion, podoplanin induces an alternative pathway of tumour cell invasion in the absence of epithelial-mesenchymal transition (EMT).

Wicki, A. et al. *Cancer Cell* 9,:261-272, 2006.

Distinct roles of vascular endothelial growth factor-D in lymphangiogenesis and metastasis

In many human carcinomas, expression of the lymphangiogenic factor vascular endothelial growth factor-D (VEGF-D) correlates with upregulated lymphangiogenesis and regional lymph node metastasis. We have employed the Rip1Tag2 transgenic mouse model of pancreatic β cell carcinogenesis to investigate the functional role of VEGF-D in the induction of lymphangiogenesis and tumour progression. Expression of VEGF-D in β cells of single-transgenic Rip1VEGF-D mice resulted in the formation of peri-insular lymphatic lacunae, often containing leukocyte accumulations and blood haemorrhages. When these mice were crossed to Rip1Tag2 mice, VEGF-D-expressing tumours also exhibited peri-tumoural lymphangiogenesis with lymphocyte accumulations and haemorrhages and frequently developed lymph node and lung metastases. Notably, tumour outgrowth and blood microvessel density were significantly reduced in VEGF-D-expressing tumours. In contrast, tumours originating from subcutaneous injection of Rip1Tag2;Rip1VEGF-D tumour cells exhibited intra-tumoural lymphangiogenesis, while hemangiogenesis was unaffected. Our results demonstrate that VEGF-D induces lymphangiogenesis, promotes metastasis to lymph nodes and lungs, and yet represses hemangiogenesis and tumour outgrowth, effects that seem to depend on the tumour microenvironment. Our study also reveals functional differences between VEGF-D and VEGF-C.

Kopfstein, L. et al. *Am. J. Pathol.* 170:1348-1361, 2007.

Placental growth factor-1 attenuates vascular endothelial growth factor-A-dependent tumour angiogenesis during β cell carcinogenesis

Members of the vascular endothelial growth factor (VEGF) family are critical players in angiogenesis and lymphangiogenesis. While VEGF-A has been demonstrated to exert fundamental functions in physiological and pathological angiogenesis, the exact role of the VEGF family member placental growth factor (PlGF) in tumour angiogenesis has remained controversial. To gain insight into PlGF function during tumour angiogenesis, we have generated transgenic mouse lines expressing human PlGF-1 in the β cells of the pancreatic islets of Langerhans (Rip1PlGF-1). In single-transgenic Rip1PlGF-1 mice, intra-insular blood vessels are found highly dilated, while islet physiology is unaffected. Upon crossing of these mice with the Rip1Tag2 transgenic mouse model of pancreatic β cell carcinogenesis, tumours of double-transgenic Rip1Tag2;Rip1PlGF-1 mice display reduced growth due to attenuated tumour angiogenesis. The co-expression of transgenic

PlGF-1 and endogenous VEGF-A in the β tumour cells of double-transgenic animals causes the formation of low-angiogenic hPlGF-1/mVEGF-A heterodimers at the expense of highly angiogenic mVEGF-A homodimers resulting in diminished tumour angiogenesis and reduced tumour-infiltration by neutrophils, known to contribute to the angiogenic switch in Rip1Tag2 mice. The results indicate that the ratio between the expression levels of two members of the VEGF family of angiogenic factors, PlGF-1 and VEGF-A, determines the overall angiogenic activity and thus the extent of tumour angiogenesis and tumour growth.

Schomber, T. et al. *Cancer Res.* 67:10840-10848, 2007.

Differential effects of the VEGF receptor inhibitor PTK787/ZK222584 on tumour angiogenesis and tumour lymphangiogenesis

Halting tumour growth by interfering with tumour-induced angiogenesis is an attractive therapeutic approach. Such treatments include humanized antibodies blocking the activity of vascular endothelial growth factor-A (VEGF-A) (Bevacizumab), soluble VEGF receptor constructs (VEGF-Trap) or small molecule inhibitors of VEGF receptor signalling, including PTK787/ZK222584 (PTK/ZK), Sorafenib, and Sunitinib. PTK/ZK, has been previously shown to specifically block VEGF-induced phosphorylation of VEGF receptor-1, 2 and 3, and thereby to inhibit endothelial cell proliferation, differentiation and tumour angiogenesis. We have investigated the effect of PTK/ZK on tumour angiogenesis and tumour lymphangiogenesis using the Rip1Tag2 transgenic mouse model of pancreatic β cell carcinogenesis. In Rip1Tag2 mice, tumour angiogenesis is predominantly mediated by VEGF-A and, as expected, PTK/ZK efficiently impaired tumour blood vessel angiogenesis and tumour growth. Double-transgenic Rip1Tag2;Rip1VEGF-C and Rip1Tag2;Rip1VEGF-D mice not only exhibit VEGF-A-dependent blood vessel angiogenesis but also tumour lymphangiogenesis induced by the transgenic expression of VEGF-C or VEGF-D. In these mouse models, PTK/ZK also repressed tumour blood vessel angiogenesis and tumour growth, yet failed to affect tumour lymphangiogenesis and lymphogenic metastasis. Adenoviral delivery of soluble VEGF receptor-3 also did not prevent tumour lymphangiogenesis in these mice. In contrast, spontaneous tumour lymphangiogenesis, as observed by the stochastic expression of VEGF-C and D in tumours of NCAM-deficient Rip1Tag2 mice, was repressed by PTK/ZK and soluble VEGF receptor-3. The results indicate that the time of onset and the levels of VEGF-C/D expression may be critical parameters in efficiently repressing tumour lymphangiogenesis and that pathways other than VEGF receptor signalling may be involved in tumour lymphangiogenesis.

Schomber, T. et al. *Mol. Cancer Ther.* 8:55-63, 2009.

Myeloid cells contribute to tumour lymphangiogenesis

The formation of new blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis) promotes tumour outgrowth and metastasis. Previously, it has been demonstrated that bone marrow-derived cells (BMDC) can contribute to tumour angiogenesis. However, the role of BMDC in lymphangiogenesis has largely remained elusive. We demonstrated by bone marrow transplantation/reconstitution and genetic lineage tracing experiments that BMDC integrate into tumour-associated lymphatic vessels in the Rip1Tag2 mouse model of insulinoma and in the TRAMP-C1 prostate cancer transplantation model. The integrated BMDC originate from the myelomonocytic lineage and trans-differentiate into lymphatic endothelial cells in tumours. Conversely, pharmacological depletion of tumour-associated macrophages reduces lymphangiogenesis. No cell fusion events are detected by genetic tracing experiments. Rather, the conversion of myeloid cells into lymphatic endothelial cells and their integration into lymphatic structures is recapitulated in two *in vitro* tube formation assays and is dependent on fibroblast

growth factor-mediated signalling. Together, the data indicate that myeloid cells can trans-differentiate into lymphatic endothelial cells and thus contribute to tumour lymphangiogenesis.

Zumsteg, A. et al. *PLoS One* 4:e7067, 2009.

VEGF receptor signalling - in control of vascular function

Vascular endothelial growth-factor receptors (VEGFRs) regulate the cardiovascular system. VEGFR1 is required for the recruitment of haematopoietic precursors and migration of monocytes and macrophages, whereas VEGFR-2 and VEGFR-3 are essential for the functions of vascular endothelial and lymphendothelial cells, respectively. Recent insights have shed light onto VEGFR signal transduction and the interplay between different VEGFRs and VEGF co-receptors in development, adult physiology and disease.

Olsson, A-K. et al. *Nat. Rev. Mol. Cell Biol*. May;7(5):359-71, 2006.

Proteomic analysis of vascular endothelial growth factor-induced endothelial cell differentiation reveals a role for chloride intracellular channel 4 (CLIC4) in tubular morphogenesis

Formation of new vessels from pre-existing capillaries demands extensive reprogramming of endothelial cells through transcriptional and post-transcriptional events. We showed that 120 protein spots in a two-dimensional isoelectric focusing/electrophoretic analysis were affected during vascular endothelial growth factor-A-induced endothelial cell tubular morphogenesis *in vitro*, as a result of changes in charge or expression level of the corresponding proteins. For about 22% of the spots, the protein products could be identified, of which several previously have been implicated in cytoskeletal reorganization and angiogenesis. One such protein was heat shock protein 27, a chaperone involved in beta-actin rearrangement that was identified as regulated in degree of serine phosphorylation. We also identified regulation of chloride intracellular channel 4 (CLIC4), the expression of which decreased during tubular morphogenesis. CLIC4 was expressed at high levels in resting vessels, whereas expression was modulated during pathological angiogenesis such as in tumour vessels. The subcellular localization of CLIC4 in endothelial cells was dependent on whether cells were engaged in proliferation or tube formation. Antisense- and small interfering RNA-mediated suppression of CLIC4 expression led to arrest in tubular morphogenesis. Our data implicate CLIC4 in formation of a vessel lumen.

Bohman, S. et al. *J. Biol. Chem.* Dec 23;280(51):42397-404, 2005.

Early lymph vessel development from embryonic stem cells

We wanted to establish a model system for lymph vessel development based on directed differentiation of murine embryonic stem cells. Stem cells were aggregated to form embryoid bodies, and subsequently cultured in 3-dimensional collagen matrix for up to 18 days. Treatment with vascular endothelial growth factor (VEGF)-C and VEGF-A individually enhanced formation of lymphatic vessel structures, although combined treatment with VEGF-C and VEGF-A was most potent and gave rise to a network of LYVE-1, podoplanin, Prox1, and VEGFR-3 positive lymphatic vessel structures running parallel to and apparently emanating from, capillaries. In contrast, fibroblast growth factor-2, hepatocyte growth factor, or hypoxia had little or no effect on the development of the early lymphatics. Further, cells of hematopoietic origin were shown to express lymphatic markers. In summary, different subpopulations of lymphatic endothelial cells were identified on the basis of differential expression of several lymphatic and blood vessel markers,

indicating vascular heterogeneity. We conclude that the present model closely mimics the early steps of lymph vessel development in mouse embryos.

Kreuger, J. et al. *Arterioscler. Thromb. Vasc. Biol.* May;26(5):1073-8, 2006.

Heparan sulphate in trans potentiates VEGFR-mediated angiogenesis

Several receptor tyrosine kinases require heparan sulphate proteoglycans (HSPGs) as coreceptors for efficient signal transduction. We have studied the role of HSPGs in the development of blood capillary structures from embryonic stem cells, a process strictly dependent on signalling via vascular endothelial growth factor receptor-2 (VEGFR-2). We show, by using chimeric cultures of embryonic stem cells defective in either HS production or VEGFR-2 synthesis, that VEGF signalling in endothelial cells is fully supported by HS expressed *in trans* by adjacent perivascular smooth muscle cells. Transactivation of VEGFR-2 leads to prolonged and enhanced signal transduction due to HS-dependent trapping of the active VEGFR-2 signalling complex. Our data imply that direct signalling via HSPG core proteins is dispensable for a functional VEGF response in endothelial cells. We propose that transactivation of tyrosine kinase receptors by HSPGs constitutes a mechanism for crosstalk between adjacent cells.

Jakobsson, L. et al. *Dev. Cell*. May;10(5):625-34, 2006.

Transcriptional profiling reveals a critical role for tyrosine phosphatase VE-PTP in regulation of VEGFR2 activity and endothelial cell morphogenesis

To define molecular events accompanying formation of the 3-dimensional (3D) vascular tube, we have characterized gene expression during vascular endothelial growth factor (VEGF)-induced tubular morphogenesis of endothelial cells. Microarray analyses were performed comparing gene induction in growth-arrested, tube-forming endothelial cells harvested from 3D collagen cultures to that in proliferating endothelial cells cultured on fibronectin. Differentially expressed genes were clustered and analyzed for specific endothelial expression through publicly available datasets. We validated the contribution of one of the identified genes, vascular endothelial protein tyrosine phosphatase (VE-PTP), to endothelial morphogenesis. Silencing of VE-PTP expression was accompanied by increased VEGFR-2 tyrosine phosphorylation and activation of downstream signalling pathways. The increased VEGFR-2 activity promoted endothelial cell cycle progression, overcoming the G(0)/G(1) arrest associated with organization into tubular structures in the 3D cultures. Proximity ligation showed close association between VEGFR-2 and VE-PTP in resting cells. Activation of VEGFR-2 by VEGF led to rapid loss of association, which was resumed with time in parallel with decreased receptor activity. In conclusion, we have identified genes, which may serve critical functions in formation of the vascular tube. One of these, VE-PTP, regulates VEGFR-2 activity thereby modulating the VEGF-response during angiogenesis.

Mellberg, S. et al. *FASEB J.* May;23(5):1490-502, 2009.

Somatic mutations in the angiopoietin-receptor TIE2 can cause both solitary and multiple sporadic venous malformations

Germline substitutions in the endothelial cell tyrosine kinase receptor TIE2 (encoded by TEK) cause a rare, inherited form of venous anomaly known as a mucocutaneous venous malformation (VMCM). We identified a somatic ‘second hit’ causing loss of function of TIE2 in a resected VMCM and assessed whether such localized, tissue-specific events have a role in the aetiology of sporadic venous malformations, which are far more common. We identified eight somatic TEK

mutations in lesions from 28 of 57 individuals (49.1%) with sporadic venous malformations; the mutations were absent from the individuals' blood and control tissues. The somatic mutations included one causing a frequent L914F substitution and several double mutations *in cis*, all of which resulted in ligand-independent TIE2 hyperphosphorylation *in vitro*. When overexpressed in human umbilical vein endothelial cells, the L914F mutant was abnormally localized and responded to ligand, in contrast to wild-type TIE2 and the common, inherited R849W mutant, suggesting that the mutations have distinct effects. The presence of the same mutations in multifocal sporadic venous malformations in two individuals suggests a common origin for the abnormal endothelial cells at the distant sites. These data show that a sporadic disease may be explained by somatic changes in a gene causing rare, inherited forms and pinpoint TIE2 pathways as potential therapeutic targets for venous malformations.

Limaye, N. et al. *Nat. Genet*. 41(1):118-124, 2009.

Recessive primary congenital lymphedema caused by a VEGFR-3 mutation

Heterozygous mutations in VEGFR-3 have been identified in some familial cases with dominantly inherited primary congenital lymphedema, known as Nonne–Milroy disease. Recessive cases of primary lymphedema with a genetic cause are not known, except for two families with syndromic hypotrichosis– lymphedema–telangiectasia, with a SOX18 mutation. We have found the first case of isolated primary congenital lymphedema with recessive inheritance, caused by a homozygous mutation in VEGFR-3. The novel mutation is a transition from alanine-to-threonine in amino acid 855, located in the ATP binding domain of the VEGFR-3 receptor. Assessment of receptor function showed impaired ligand induced internalisation and ERK1/2 activity. Moreover, receptor phosphorylation was reduced, although less so than for a kinase-dead VEGFR-3 mutation, which causes Nonne–Milroy disease. We conclude that a hypomorphic VEGFR-3 mutation, with moderate effect on receptor function, in a homozygous state, can result in insufficient lymphatic functioning. Thus, in addition to Nonne–Milroy disease with dominant inheritance, VEGFR-3 alterations can cause isolated recessive primary congenital lymphedema. These data expand our understanding of the aetiology of congenital lymphedema and suggest that large scale screening of VEGFR-3 in all primary lymphedema patients is necessary.

Ghalamkarpour, A. et al. *J. Med. Genet.* 46(6):399-404, 2009.

Sporadic *in utero* generalized oedema associated with mutations in the lymphangiogenic genes VEGFR3 and FOXC2

Hydrops fetalis is defined as generalized subcutaneous oedema with an effusion in at least one body cavity and has a high mortality rate. The most common associated diagnoses are congenital heart disease, arrhythmias, twin-to-twin transfusion, and congenital anomalies such as skeletal dysplasias, chromosomal abnormalities, congenital viral infection, and congenital anaemia. The aetiology is unknown for 25% of the patients. There have been reports of hydrops fetalis occurring in families with Nonne-Milroy disease, lymphedema-distichiasis, and hypotrichosis-lymphedema-telangiectasia syndrome, associated with mutations in the three lymphangiogenic genes, VEGFR-3, FOXC2, and SOX18, respectively. This led us to hypothesize that *de novo* mutations in these genes could explain sporadic occurrences of foetal oedema. To test this, we screened the three genes in 12 patients. In three of the patients, we identified a mutation: two in VEGFR-3 and one in FOXC2.

Two of the mutations were *de novo* and one was either *de novo* or non-penetrant inherited. In these patients, the generalized oedema resorbed spontaneously, either *in utero* or after birth. In the two individuals with a VEGFR-3 mutation, oedema remained limited to lower limbs.
We conclude that mutations in the VEGFR-3 and FOXC2 genes account for a subset of patients with unexplained *in utero* generalized subcutaneous oedema and hydrops fetalis without family history of lymphedema. Lymphangiogenic genes should be screened for mutations in sporadic patients diagnosed with foetal oedema.

Ghalamkarpour, A. et al. *J. Pediatrics*, 9:90-93, 2009.

Parkes Weber syndrome, vein of Galen aneurysmal malformation, and other fast-flow vascular anomalies and specific neural tumours associated with RASA1 mutations

Capillary malformation-arteriovenous malformation (CM-AVM) is a newly recognized autosomal dominant disorder, caused by mutations in the RASA1 gene in six families. We studied 42 novel RASA1 mutations and the associated phenotype in 44 families. The penetrance and *de novo* occurrence were high. All affected individuals presented multifocal capillary malformations (CMs), which represent the hallmark of the disorder. Importantly, one-third had fast-flow vascular lesions. Among them, we observed severe intracranial AVMs, including vein of Galen aneurysmal malformation, which were symptomatic at birth or during infancy, extracranial AVM of the face and extremities, and Parkes Weber syndrome (PKWS), previously considered sporadic and non-genetic. These fast-flow lesions can be differed from the other two genetic AVMs seen in hereditary hemorrhagic telangiectasia (HHT) and in phosphatase and tensin homolog (PTEN) hamartomatous tumour syndrome. Finally, some CM-AVM patients had neural tumours reminiscent of neurofibromatosis type 1 or 2. This is the first extensive study on the phenotypes associated with RASA1 mutations, and unravels their wide heterogeneity.

Revencu, N. et al. *Hum. Mutat.* 29(7):959-65, 2008.

PTHR1 mutations associated with Ollier disease result in receptor loss of function

PTHR1 signalling pathway is critical for regulation of endochondral ossification. Thus, abnormalities in genes belonging to this pathway could potentially participate in the pathogenesis of Ollier disease/Maffucci's syndrome, two developmental disorder defined by the presence of multiple enchondromas. In agreement, a functionally deleterious mutation in PTHR1 (p.R150C) was identified in enchondromas from two of six unrelated patients with enchondromatosis. However, neither the p.R150C mutation (26 tumours) nor any other mutation in the PTHR1 gene (11 patients) could be identified in another study. To further define the role of PTHR1 signalling pathway in Ollier disease and Maffucci syndrome, we analysed the coding sequences of four genes (PTHR1, IHH, PTHrP and GNAS1) in leukocyte and/or tumour DNA from 57 and 23 patients affected with Ollier disease or Maffucci syndrome respectively. We identified three previously unknown missense mutations in PTHR1 in patients with Ollier disease at the heterozygous state. Two mutations (p.G121E, p.A122T) were present only in enchondromas, and one (p.R255H) in both enchondroma and leukocyte DNA. Assessment of receptor function demonstrated that these three mutations impair PTHR1 function, either by reducing the affinity of the receptor for PTH or reducing receptor expression at the cell surface. These mutations were not found in DNA from 222 controls. Including our data, PTHR1 functionally deleterious mutations have now been identified in 5/31 enchondromas from Ollier patients. These findings provide further support for the idea that heterozygous mutations in PTHR1 that impair receptor function participate in the pathogenesis of Ollier disease in some patients.

Couvineau, A. et al. *Hum. Mol. Genet.* 17(18):2766-75, 2008.

VEGF-D deficiency in mice does not affect embryonic or postnatal lymphangiogenesis but reduces lymphatic metastasis

Vascular endothelial growth factor-D (VEGF-D) is one of the two ligands of the VEGFR-3 receptor on lymphatic endothelial cells. Gene-silencing studies in mice and *Xenopus* tadpoles recently showed that the role of endogenous VEGF-D in lymphatic development is moderate. By contrast, exogenous VEGF-D is capable of stimulating lymphangiogenesis. Nonetheless, its endogenous role in pathological conditions remains largely unknown. We reassessed its role in disease, using *Vegf-d*^{null} mice. *Vegf-d*^{null} mice were generated, which under physiological conditions, displayed normal embryonic and postnatal lymphangiogenesis and lymphatic remodelling, efficient lymphatic functioning and normal health. *Vegf-d*^{null} mice also responded normally in models of skin wound healing and healing of infarcted myocardium, despite enhanced expression of VEGF-D in these models in wild type mice. In contrast, *Vegf-d*^{null} mice displayed reduced peritumoural lymphangiogenesis and lymph node metastasis in an orthotopic pancreatic tumour model. Together, our data indicate that endogenous VEGF-D in mice is dispensable for lymphangiogenesis during development, in postnatal and adult physiology and in several pathological conditions, but significantly contributes to lymphatic metastasis.

Koch, M. et al, *J. Pathol.* 219:356-364, 2009.

Role of VEGF-D and VEGFR-3 in developmental lymphangiogenesis, a chemicogenetic study in *Xenopus* tadpoles

The importance of the lymphangiogenic factor VEGF-D and its receptor VEGFR-3 in early lymphatic development remains largely unresolved. We therefore investigated their role in *Xenopus laevis* tadpoles, a small animal model allowing chemicogenetic dissection of developmental lymphangiogenesis. Single morpholino antisense oligo knockdown of xVEGF-D did not affect lymphatic commitment, but transiently impaired lymphatic endothelial cell (LEC) migration. Notably, combined knockdown of xVEGF-D with xVEGF-C at suboptimal morpholino concentrations resulted in more severe migration defects and lymphedema formation than the corresponding single knockdowns. Knockdown of VEGFR-3 or treatment with the VEGFR-3 inhibitor MAZ51 similarly impaired lymph vessel formation and function and caused pronounced oedema. VEGFR-3 silencing by morpholino knockdown, MAZ51 treatment, or xVEGF-C/D double knockdown also resulted in dilation and dysfunction of the lymph heart. These findings document a critical role of VEGFR-3 in embryonic lymphatic development and function, and reveal a previously unrecognized modifier role of VEGF-D in the regulation of embryonic lymphangiogenesis in frog embryos.

Ny, A. et al, *Blood* 112:1740-1749, 2008.

Blood flow remodels growing vasculature during vascular endothelial growth factor gene therapy and determines between capillary arterialization and sprouting angiogenesis

For clinically relevant proangiogenic therapy, it would be essential that the growth of the whole vascular tree is promoted. Vascular endothelial growth factor (VEGF) is well known to induce angiogenesis, but its capability to promote growth of larger vessels is controversial. We hypothesized that blood flow remodels vascular growth during VEGF gene therapy and may contribute to the growth of large vessels. Adenoviral (Ad) VEGF or LacZ control gene transfer was performed in rabbit hind limb semimembranous muscles with or without ligation of the profound

femoral artery (PFA). Contrast-enhanced ultrasound and dynamic susceptibility contrast MRI demonstrated dramatic 23- to 27-fold increases in perfusion index and a strong decrease in peripheral resistance six days after AdVEGF gene transfer in normal muscles. Enlargement by 20-fold, increased pericyte coverage, and decreased alkaline phosphatase and dipeptidyl peptidase IV activities suggested the transformation of capillaries toward an arterial phenotype. Increase in muscle perfusion was attenuated, and blood vessel growth was more variable, showing more sprouting angiogenesis and formation of blood lacunae after AdVEGF gene transfer in muscles with ligated PFA than in normal muscles. Three-dimensional ultrasound reconstructions and histology showed that the whole vascular tree, including large arteries and veins, was enlarged manifold by AdVEGF. Blood flow was normalized and enlarged collaterals persisted in operated limbs 14 days after AdVEGF treatment. This study shows that (1) blood flow modulates vessel growth during VEGF gene therapy and (2) VEGF overexpression promotes growth of arteries and veins and induces capillary arterialization leading to supraphysiological blood flow in target muscles.

Rissanen, T. et al. *Circulation* 112:3937-3946, 2005.

Short and long-term effects of hVEGF-A165 in Cre-activated transgenic mice

We have generated a transgenic mouse where hVEGF-A(165) expression has been silenced with loxP-STOP fragment, and we used this model to study the effects of hVEGF-A(165) over-expression in mice after systemic adenovirus mediated Cre-gene transfer. Unlike previous conventional transgenic models, this model leads to the expression of hVEGF-A(165) in only a low number of cells in the target tissues in adult mice. Levels of hVEGF-A(165) expression were moderate and morphological changes were found mainly in the liver, showing typical signs of active angiogenesis. Most mice were healthy without any major consequences up to 18 months after the activation of hVEGF-A(165) expression. However, one mouse with a high plasma hVEGF-A(165) level died spontaneously because of bleeding into abdominal cavity and having liver haemangioma, haemorrhagic paratubarian cystic lesions and spleen peliosis. Also, two mice developed malignant tumours (hepatocellular carcinoma and lung adenocarcinoma), which were not seen in control mice. We conclude that long-term uncontrolled hVEGF-A(165) expression in only a limited number of target cells in adult mice can be associated with pathological changes, including possible formation of malignant tumours and uncontrolled bleeding in target tissues. These findings have implications for the design of long-term clinical trials using hVEGF-A(165) gene and protein.

Leppänen, P. et al. *PLoS ONE 1*:e13, 2006.

Vascular endothelial growth factor-A and platelet-derived growth factor-B combination gene therapy prolongs angiogenic effects via recruitment of interstitial mononuclear cells and paracrine effects rather than improved pericyte coverage of angiogenic vessels

New revascularization therapies are urgently needed for patients with severe coronary heart disease who lack conventional treatment options. We describe a new proangiogenic approach for these no-option patients using adenoviral (Ad) intramyocardial vascular endothelial growth factor (VEGF)-B186 gene transfer, which induces myocardium-specific angiogenesis and arteriogenesis in pigs and rabbits. After acute infarction, AdVEGF-B186 increased blood vessel area, perfusion, ejection fraction, and collateral artery formation and induced changes toward an ischemia-resistant myocardial phenotype. Soluble VEGF receptor-1 and soluble neuropilin receptor-1 reduced the effects of AdVEGF-B186, whereas neither soluble VEGF receptor-2 nor inhibition of nitric oxide production had this result. The effects of AdVEGF-B186 involved activation of neuropilin receptor-1, which is highly expressed in the myocardium, via recruitment of G-protein-alpha interacting

protein, terminus C (GIPC) and upregulation of G-protein-alpha interacting protein. AdVEGF-B186 also induced an antiapoptotic gene expression profile in cardiomyocytes and had metabolic effects by inducing expression of fatty acid transport protein-4 and lipid and glycogen accumulation in the myocardium. VEGF-B186 displayed strikingly distinct effects compared with other VEGFs. These effects may be mediated at least in part via a G-protein signalling pathway. Tissue-specificity, high efficiency in ischemic myocardium, and induction of arteriogenesis and antiapoptotic and metabolic effects make AdVEGF-B186 a promising candidate for the treatment of myocardial ischemia.

Korpisalo, P. et al. *Circ. Res***.** 103:1092-1099, 2008.

VEGF-D transgenic mice show enhanced blood capillary density, improved post-ischemic muscle regeneration and increased susceptibility to tumour formation

Vascular endothelial growth factor-D (VEGF-D) has angiogenic and lymphangiogenic activity, but its biologic role has remained unclear because knockout mice showed no clear phenotype. Transgenic (TG) mice expressing the mature form of human VEGF-D (hVEGF-D) were produced by lentiviral (LV) transgenesis using the perivitelline injection method. Several viable founders showed a macroscopically normal phenotype and the transgene transmitted through germ line. Expression of hVEGF-D mRNA was high in skeletal muscles, skin, pancreas, heart, and spleen. A significant increase was found in capillary density of skeletal muscles and myocardium, whereas no changes were observed in lymphatic capillary density. After induction of hind limb ischemia, the TG mice showed enhanced capacity for muscle regeneration. However, on aging the TG mice had significantly increased mortality from malignant tumours, of which half were breast adenocarcinomas characterized with the absence of periductal muscle cells. Some tumours metastasized into the lungs. In addition, lung and skin tumours were found, but no blood- or lymphatic vessel-derived malignancies were detected. We conclude that in mice hVEGF-D is an angiogenic factor associated with improved muscle regeneration after ischemic injury but also with increased incidence of tumour formation with a preference for mammary gland tumours.

Kärkkäinen, A-M. et al. *Blood* 113:4468-4475, 2009.

Vascular endothelial growth factor-B induces myocardium-specific angiogenesis and arteriogenesis via vascular endothelial growth factor receptor-1 and Neuropilin receptor-1 dependent mechanisms

New revascularization therapies are urgently needed for patients with severe coronary heart disease who lack conventional treatment options. We describe a new proangiogenic approach for these no-option patients using adenoviral (Ad) intramyocardial vascular endothelial growth factor (VEGF)-B186 gene transfer, which induces myocardium-specific angiogenesis and arteriogenesis in pigs and rabbits. After acute infarction, AdVEGF-B186 increased blood vessel area, perfusion, ejection fraction, and collateral artery formation and induced changes toward an ischemia-resistant myocardial phenotype. Soluble VEGF receptor-1 and soluble neuropilin receptor-1 reduced the effects of AdVEGF-B186, whereas neither soluble VEGF receptor-2 nor inhibition of nitric oxide production had this result. The effects of AdVEGF-B186 involved activation of neuropilin receptor-1, which is highly expressed in the myocardium, via recruitment of G-protein-alpha interacting protein, terminus C (GIPC) and upregulation of G-protein-alpha interacting protein. AdVEGF-B186 also induced an antiapoptotic gene expression profile in cardiomyocytes and had metabolic effects by inducing expression of fatty acid transport protein-4 and lipid and glycogen accumulation in the myocardium. VEGF-B186 displayed strikingly distinct effects compared with other VEGFs. These effects may be mediated at least in part via a G-protein signalling pathway. Tissue-specificity, high

efficiency in ischemic myocardium, and induction of arteriogenesis and antiapoptotic and metabolic effects make AdVEGF-B186 a promising candidate for the treatment of myocardial ischemia.

Lähteenvuo, J.E. et al. *Circulation* 119:845-856, 2009.

Spheroid-based engineering of a human vasculature in mice

The complexity of the angiogenic cascade limits cellular approaches to studying angiogenic endothelial cells (ECs). In turn, *in vivo* assays do not allow the analysis of the distinct cellular behaviour of ECs during angiogenesis. We have shown that ECs can be grafted as spheroids into a matrix to give rise to a complex three-dimensional network of human neovessels in mice. The grafted vasculature matures and is connected to the mouse circulation. The assay is highly versatile and facilitates numerous applications including studies of the effects of different cytokines on angiogenesis. Modifications make it possible to study human lymphangiogenic processes *in vivo*. EC spheroids can also be co-implanted with other cell types for tissue engineering purposes.

Alajati, A. et al., *Nat. Methods,* 5:439-45, 2008.

Tumour stroma marker endosialin (Tem1) is a binding partner of metastasis-related protein Mac-2 BP/90K

Tumour development involves complex bidirectional interactions between tumour cells and host stromal cells. Endosialin (Tem1) has been identified as a highly O-glycosylated transmembrane glycoprotein, which is specifically expressed by tumour vessel-associated pericytes and stromal fibroblasts of a wide range of human tumours. Recent experiments in endosialin-deficient mice have unravelled a critical role of endosialin in site-specific tumour progression and metastasis. To molecularly understand the mechanisms of endosialin function, we aimed to identify extracellular endosialin ligands and identified Mac-2 BP/90K as a specific interaction partner. Detailed biochemical analyses identified a C-terminal fragment of Mac-2 BP/90K, which was shown to contain binding sites for galectin-3, and collagens as the structures responsible for endosialin binding. Subsequent expression analysis of Mac-2 BP/90K *in vivo* revealed weak or no expression in most normal tissues and strong up-regulation in tumour cells of human neoplastic tissues. Intriguingly, the expression patterns of Mac-2 BP/90K and endosialin were mutually exclusive in all human tissues. Correspondingly, loss-of-function adhesion experiments of Mac-2 BP/90K-expressing tumour cells on endosialin-expressing fibroblasts revealed a repulsive outcome of the Mac-2 BP/90K interaction. Taken together, the experiments identify a novel repulsive interaction between endosialin on stromal fibroblasts and Mac-2 BP/90K on tumour cells.

Becker, R. et al. *FASEB J,* 22:3059-67, 2008.

Endosialin (Tem1) is a marker of tumour-associated myofibroblasts and tumour vessel-associated mural cells

Endosialin (Tem1) has been identified by two independent experimental approaches as an antigen of tumour-associated endothelial cells, and it has been claimed to be the most abundantly expressed tumour endothelial antigen, making it a prime candidate for vascular targeting purposes. Recent experiments have challenged the endothelial expression of endosialin and suggested an expression by activated fibroblasts and pericytes. Thus, clarification of the controversial cellular expression of endosialin is critically important for an understanding of its role during tumour progression and its validation as a potential therapeutic target. We have therefore performed extensive expression profiling analyses of endosialin. The experiments unambiguously demonstrate that endosialin is expressed by tumour-associated myofibroblasts and mural cells and not by endothelial cells. Endosialin expression is barely detectable in normal human tissues with moderate expression only

detectable in the stroma of the colon and the prostate. Corresponding cellular experiments confirmed endosialin expression by mesenchymal cells and indicated that it may in fact be a marker of mesenchymal stem cells. Silencing endosialin expression in fibroblasts strongly inhibited migration and proliferation. Collectively, the experiments validate endosialin as a marker of tumour-associated myofibroblasts and tumour vessel-associated mural cells. The data warrant further functional analysis of endosialin during tumour progression and its exploitation as marker of tumour vessel-associated mural cells, expression of which may reflect the non-normalized phenotype of the tumour vasculature.

Christian, S. et al., *Am. J. Pathol.* 172:486-94, 2008.

The sialomucin CD34 is a marker of lymphatic endothelial cells in human tumours

The mechanisms of lymphangiogenesis have been increasingly understood in recent years. Yet, the contribution of lymphangiogenesis versus lymphatic cooption in human tumours and the functionality of tumour lymphatics are still controversial. Furthermore, despite the identification of lymphatic endothelial cell (LEC) markers such as Prox1, podoplanin, LYVE-1, and VEGFR-3, no activation marker for tumour-associated LECs has been identified. Applying double-staining techniques with established LEC markers, we have screened endothelial cell differentiation antigens for their expression in LECs. These experiments identified the sialomucin CD34 as being exclusively expressed by LECs in human tumours but not in corresponding normal tissues. CD34 is expressed by LYVE-1(+)/podoplanin(+)/Prox1(+) tumour-associated LECs in colon, breast, lung, and skin tumours. More than 60% of analyzed tumours contained detectable intratumoural lymphatics. Of these, more than 80% showed complete co-localization of CD34 with LEC markers. In contrast, LECs in all analyzed normal organs did not express CD34. Corresponding analyses of experimental tumours revealed that mouse tumour-associated LECs do not express CD34. Taken together, these experiments identify CD34 as the first differentially expressed LEC antigen that is selectively expressed by tumour-associated LECs. The data warrant further exploration of CD34 in tumour-associated LECs as a prognostic tumour marker.

Fiedler, U. et al., *Am. J. Pathol.* 168:1045-53, 2006

Endothelial ephrinB2 is controlled by microenvironmental determinants and associates context-dependently with CD31

The EphB ligand ephrinB2 has been identified as a critical determinant of arterial endothelial differentiation and as a positive regulator of invading endothelial cells during angiogenesis. We have aimed at identifying determinants of endothelial cell ephrinB2 expression. Arteriovenous asymmetrical endothelial cell ephrinB2 expression *in vivo* is lost on transfer into culture with aortic endothelial cells becoming partially ephrinB2-negative and saphenous vein endothelial cells becoming partially ephrinB2-positive. Contact with smooth muscle cells and angiogenic stimulation by vascular endothelial growth factor lead to an increased endothelial cell ephrinB2 expression. Quiescent, smooth muscle-contacting endothelial cells express ephrinB2 uniformly on their luminal surface. In contrast, monolayer endothelial cells translocate ephrinB2 to interendothelial cell junctions, which is strongly enhanced by EphB4-Fc-mediated receptor body activation. Junctional ephrinB2 colocalizes and coimmunoprecipitates with CD31. This study identifies distinct regulatory mechanisms of endothelial ephrinB2 expression and cellular distribution in quiescent and activated endothelial cells. The data demonstrate that endothelial cell ephrinB2 expression is controlled by microenvironmental determinants rather than being an intrinsic endothelial cell differentiation marker.

Korff, T. et al. *Arterioscler. Thromb. Vasc. Biol.* 26:468-74, 2006.

Discovery of microvascular miRNAs using public gene expression data: miR-145 is expressed in pericytes and is a regulator of Fli1

A function for the microRNA (miRNA) pathway in vascular development and angiogenesis has been firmly established. miRNAs with selective expression in the vasculature are attractive as possible targets in miRNA-based therapies. However, little is known about the expression of miRNAs in microvessels *in vivo*. We identified candidate microvascular-selective miRNAs by screening public miRNA expression datasets. Bioinformatics predictions of microvascular-selective expression were validated with real-time quantitative reverse transcription PCR on purified microvascular fragments from mouse. Pericyte expression was shown with *in situ* hybridization on tissue sections. Target sites were identified with 3' UTR luciferase assays, and migration was tested in a microfluid chemotaxis chamber. miR-145, miR-126, miR-24, and miR-23a were selectively expressed in microvascular fragments isolated from a range of tissues. *In situ* hybridization and analysis of Pdgfb retention motif mutant mice demonstrated predominant expression of miR-145 in pericytes. We identified the Ets transcription factor Friend leukaemia virus integration 1 (Fli1) as a miR-145 target, and showed that elevated levels of miR-145 reduced migration of microvascular cells in response to growth factor gradients *in vitro*. Thus, miR-126, miR-24 and miR-23a are selectively expressed in microvascular endothelial cells *in vivo*, whereas miR-145 is expressed in pericytes. miR-145 targets the hematopoietic transcription factor Fli1 and blocks migration in response to growth factor gradients. Our findings have implications for vascular disease and provide necessary information for future drug design against miRNAs with selective expression in the microvasculature.

Larsson, E. et al. *Genome Med*. Nov 16;1(11):108, 2009.

Mutually exclusive gene modules define the diversity of smooth muscle

Smooth muscle cells (SMCs) are key components of all hollow organs, where they perform contractile, synthetic and other functions. Unlike other muscle cells, SMCs are not terminally differentiated, but exhibit considerable phenotypic variation. Such variation is manifested both across disease states such as asthma and atherosclerosis, and physiological states such as pregnancy and wound healing. While there has been considerable investigation into the diversity of SMCs at the level of morphology and individual biomarkers, less is known about the diversity of SMCs at the level of the transcriptome. To explore this question, we performed an extensive statistical analysis that integrates 200 transcriptional profiles obtained in different SMC phenotypes and reference tissues. Our results point towards a non-trivial hypothesis: that transcriptional variation in different SMC phenotypes is characterized by coordinated differential expression of two mutually exclusive (anti-correlating) gene modules. The first of these modules (C) encodes 19 co-transcribed cell cycle associated genes, whereas the other module (E) encodes 41 co-transcribed extra-cellular matrix components. We propose that the positioning of smooth muscle cells along the C/E axis constitutes an important determinant of SMC phenotypes. In conclusion, our study introduces a new approach to assess phenotypic variation in smooth muscle cells, and is relevant as an example of how integrative bioinformatics analysis can shed light on not only terminal differentiated states but also subtler details in phenotypic variability. It also raises the broader question whether coordinated expression of gene modules is a common mechanism underlying phenotypic variability in mammalian cells.

Larsson, E. et al. *Mol. Genet. Genomics* Aug;280(2):127-37, 2008.

HeliCis: a DNA motif discovery tool for colocalized motif pairs with periodic spacing

Correct temporal and spatial gene expression during metazoan development relies on combinatorial interactions between different transcription factors. As a consequence, *cis*-regulatory elements often colocalize in clusters termed *cis*-regulatory modules. These may have requirements on organizational features such as spacing, order and helical phasing (periodic spacing) between binding sites. Due to the turning of the DNA helix, a small modification of the distance between a pair of sites may sometimes drastically disrupt function, while insertion of a full helical turn of DNA (10-11 bp) between *cis* elements may cause functionality to be restored. Recently, *de novo* motif discovery methods, which incorporate organizational properties such as colocalization and order preferences, have been developed, but there are no tools which incorporate periodic spacing into the model. We have developed a web based motif discovery tool, HeliCis, which features a flexible model, which allows de novo detection of motifs with periodic spacing. Depending on the parameter settings it may also be used for discovering colocalized motifs without periodicity or motifs separated by a fixed gap of known or unknown length. We show on simulated data that it can efficiently capture the synergistic effects of colocalization and periodic spacing to improve detection of weak DNA motifs. It provides a simple to use web interface, which interactively visualizes the current settings and thereby makes it easy to understand the parameters and the model structure. HeliCis thus provides simple and efficient de novo discovery of colocalized DNA motif pairs, with or without periodic spacing. Our evaluations show that it can detect weak periodic patterns which are not easily discovered using a sequential approach, i.e. first finding the binding sites and second analyzing the properties of their pairwise distances.

Larsson, E. et al. *BMC Bioinformatics* Oct 28;8(1):418, 2007.

Microarray analysis of blood microvessels from PDGF-B and PDGF-Rbeta mutant mice identifies novel markers for brain pericytes

Normal blood microvessels are lined by pericytes, which contribute to microvessel development and stability through mechanisms that are poorly understood. Pericyte deficiency has been implicated in the pathogenesis of microvascular abnormalities associated with diabetes and tumours. However, the unambiguous identification of pericytes is still a problem because of cellular heterogeneity and few available molecular markers. We have described an approach to identify pericyte markers based on transcription profiling of pericyte-deficient brain microvessels isolated from platelet-derived growth factor (PDGF-B)-/- and PDGF beta receptor (PDGFRbeta)-/- mouse mutants. The approach was validated by the identification of known pericyte markers among the most down-regulated genes in PDGF-B-/- and PDGFRbeta-/- microvessels. Of candidates for novel pericyte markers, we selected ATP-sensitive potassium-channel Kir6.1 (also known as Kcnj8) and sulfonylurea receptor 2, (SUR2, also known as Abcc9), both part of the same channel complex, as well as delta homologue 1 (DLK1) for *in situ* hybridization, which demonstrated their specific expression in brain pericytes of mouse embryos. We also show that Kir6.1 is highly expressed in pericytes in brain but undetectable in pericytes in skin and heart. The three new brain pericyte markers are signalling molecules implicated in ion transport and intercellular signalling, potentially opening new windows on pericyte function in brain microvessels.

Bondjers, C. et al. *FASEB J.* Aug;20(10):1703-5, 2006.

2. Dissemination and use

Publishable results of the Final plan for using and disseminating the knowledge.