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Arteriogenesis – Molecular Regulation, Pathophysiology and Therapeutics I

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FOREWORD

Anecdotal observations about the existence of a bypass circulation in the presence of an arterial obstruction, spontaneous or induced, exist since a long time. It may have started in the Royal deer park of King George where Dr. Hunter occluded vessels feeding the deer's antler and where the site of occlusion was bypassed by collateral vessels without disturbing the growth of the majestic trophy. Centuries later post mortem studies in humans showed that most hearts are endowed with collateral vessels even in the absence of arterial occlusions, notably the human heart. The descriptive part of research culminated in the work of Fulton [1, 2] who showed the marked enlargement of pre-existing collaterals in human hearts in the presence of arterial occlusive disease. Dr. Fultons impressive angiograms were, apart from his scientific insights, the results of technical refinements, i.e., the use of a contrast material with high density for "soft" X-rays and 3-D viewing. About a decade later in-vivo coronary angiography took off and showed for the first time coronary collaterals in the beating heart, and cases were reported where these vessels had enlarged just in time to prevent an infarct. The important life and tissue-saving role of collaterals was questioned, again a decade later, when angiographers showed that the degree of collateralization correlated with the degree of coronary disease [3, 4], and the erroneous conclusion was drawn that collaterals were rather a bad omen for the downhill course of the disease. This was finally straightened out by studies showing the beneficial effect of collaterals [5]. Basic science became interested in the mechanisms of vascular growth and studies on the rate of growth, the functional capacity, the structure and ultra-structure appeared, because it was obvious that this new knowledge was potentially useful to design new types of therapy, which would benefit patients [6]. However, the interest in vascular research suffered setbacks by allegedly easier and more profitable types of therapy that consisted also of bypasses but this time produced by surgeons, or by eliminating occlusions and stenoses by balloon dilatation and stent deployment at the hands of cardiologists. Only when the stents became occluded, interest in vascular research soared again [7]. Another impediment to "Arteriogenesis", as research into collateral vessel development was now called, was the flood of publications that followed the claim by Dr. Folkman [8] that with the advent of vascular growth factor the scourges of mankind, i.e., arteriosclerosis and cancer, could be cured by application of one single principle: angiogenesis and its antidote, anti-angiogenesis. Since this almost coincided with the discovery that genetic targeting of the newly discovered vascular endothelial growth factor (VEGF) was embryonic lethal [9], research in endothelial biology received an enormous boost, almost completely neglecting the fact that, at least in arterial disease with tissue ischemia, no shortage of endothelial cells exists and that diseased arteries cannot be replaced by capillaries. Clinical studies using vascular growth factors remained inconclusive in arterial disease [10, 11] and growth factors were not included into the armamentarium of standard therapies. Antiangiogenic therapy in cancer did also not live up to initial expectations [12].

In spite of these impediments arteriogenesis has made great strides forward and we know now that bone marrow derived cells, in particular monocytes, play an essential role [13, 14]. We know now the principal players in the field, thanks to the availability of genetically altered mice. We know that increased fluid shear stress [15, 16], as it occurs in pre-existent arteriolar collaterals as a consequence of an acute occlusion, is the primary physical stimulus, which is responded to by changes in the activity of numerous ion channels, in the up-regulation of NO-producing enzymes [17] and in gene expression. We do not know enough about the molecular signaling that originates from the activated endothelium to the smooth muscles in the media, the major players in arteriogenesis, because their multiplication and remodeling results in the vessel enlargement by a factor of up to 10-fold its original diameter and tissue mass (depending on species and organ supplied and size of occluded artery). The smooth muscles of the media, stimulated somehow by signals from the activated endothelium, produce monocyte chemoattracting protein-1 (MCP-1) [18], which is responsible for the accumulation of monocytes in the wider adventitial space of the developing vessel. Surprisingly, NO plays also a defining role [17]. Surprising, because NO is an anti-mitogen for smooth muscles [19], the most important players in arteriogenesis. However, L-NAME, a non-specific inhibitor of

all NO producing enzymes, prevents arteriogenesis and the double knock-out of eNOS+iNOS seconds that.

Can we now hope that we have come closer to the development of a new therapeutic principle applicable to human patients? Yes, we can! However, we have to overcome strong competition and perhaps even adversaries. Any new drug or vector has to be superior to the well-established revascularization procedures of the surgeons and cardiologists. Industry has to be convinced that it is worthwhile to invest into vascular research again. Again, because most pharmaceutical companies had closed their labs for cardiovascular research in favor of the development of high priced anti-cancer drugs. However, there is hope, but only when we succeed in the complete unraveling of the pathways that rule the development of arterial bypasses, which would enable us to find the limiting step that had escaped evolutionary selection pressure and that we have to stimulate to better Mother Nature.

Wolfgang Schaper

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PREFACE

Arteriogenesis describes the growth, remodelling and maturation of pre-existing collateral arteries into true arteries. Functioning as natural bypasses these collaterals are able to compensate for nonfunctional stenotic/occluded arteries and, therefore, may, in the hopefully not too distant future, substitute for clinical interventions such as balloon dilatation or even heart transplantation. Pre-existing collateral arteries, the growth of which is triggered by increased mechanical stress, occur not only in the heart, but virtually in every organ of the body. Research on molecular mechanisms of arteriogenesis aiming to comprehend this naturally occurring physiological process in order to induce it therapeutically where needed, started several years ago. A first book reporting basic and current knowledge on arteriogenesis was released in 2004 ("Arteriogenesis", Wolfgang Schaper and Jutta Schaper (eds.)). Since then, a lot of progress has been made on understanding how mechanical stress (fluid shear stress) is translated into the diverse biological signal transduction cascades finally resulting in cell proliferation and arterial growth. With the publication of this volume, we want to update the current knowledge in collateral artery growth.

Wolfgang Schaper gives an overview of the basics and focuses on the mechanistic role of physical forces, the innate immune system and nitric oxide; Kerstin and Christian Troidl report on the function of Ca^{2+} and Ca^{2+} channels, as well as on downstream signaling mechanisms. Due to the pro- as well as anti-arteriogenic properties of nitric oxide, the involvement of the NO-system in arteriogenesis has been heavily discussed for several years and Joseph Unthank depicts in detail the influences of NO and reactive oxygen species on vascular remodeling. In a more systemic approach, Paul Quax and Vincent van Weel report on the role of bone-marrow-derived cells. Elisabeth Deindl discusses epigenetic influences; James Faber gives insight into genetic and environmental mechanisms controlling the formation and maintenance of collateral arteries; Ferdinand Le Noble addresses the molecular pathway controlling arteriolar branching in the developing embryo and Florian Limbourg summarizes the role of the Notch signaling pathway for arterial cell types in embryonic development and postnatal life.

While the first part of this book concentrates more on the molecular mechanisms of collateral artery growth, involving also the embryonic development of collaterals, the second part deals with the current clinical situation and therapeutically approaches. Niels van Royen illustrates the relevance of collateral arteries in patients with vascular diseases and reviews the therapeutic options to stimulate arteriogenesis. Ivo and Eva Buschmann delineate the function of external counterpulsation and Christian Kupatt and Teresa Trenkwalder present the features of thymosin beta4. Finally, Imo Hoefer introduces novel intra-arterial delivery platforms defined to enable clinicians to promote arteriogenesis locally. In summary, this volume aims to provide a comprehensive update of the current knowledge in the challenging and increasingly important field of arteriogenesis continuing a series in the timely form of an e-book. The story begins now…

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by S. Leese

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Chapter 1. Genetic and Environmental Mechanisms Controlling Formation and Maintenance of the Native Collateral Circulation

James E. Faber
Xuming Dai
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Abstract: Most tissues have arteriole-like vessels that cross-connect one or more of the outer-most branches of opposing arterial trees. These pre-existing "native collaterals" have unique characteristics and functions—as unique from vessels of the general and lymphatic circulations as the latter are from each other. Rare in number, these vessels somehow defy the developmental patterning cues that assure that branching proceeds from arteriole-to-capillaries-to-venule in the general circulation. As a consequence, blood flow and shear stress in collaterals of healthy tissues are exceedingly low and oscillatory, and circumferential wall stress is high, relative to elsewhere in the microcirculation. Yet these vessels persist in this unusual hemodynamic environment—one that favors inflammation, hemostasis and vessel pruning elsewhere in the general circulation. Their persistence is crucial: When blood flow to an arterial tree is reduced or blocked by acute or chronic obstructive disease, native collaterals serve as endogenous bypass vessels, providing retrograde perfusion from the adjacent unobstructed tree. The amount of perfusion and thus ischemic injury is initially dependent on the extent (density and diameter) of these vessels, and subsequently on their ability to anatomically enlarge, a process termed "arteriogenesis". Despite the uniqueness and importance of the collateral circulation, until recently almost nothing was known about when or how it forms, ie, "collaterogenesis". Moreover, evidence suggests that the extent of this circulation varies widely among healthy individuals. Yet only recently have the responsible mechanisms begun to be examined. This chapter will review recent work that is beginning to answer these questions and also to identify how genetic and environmental factors govern formation and maintenance of the native collateral circulation.

Introduction: Collaterals -- the "Third" Circulation

In recent decades much has been learned about the mechanisms governing formation of the *General* arterial-venous and *Lymphatic* circulations. These circulations are uniquely different, with regard to certain differentiation molecules and morphogenic processes that mediate their development, as well as in their anatomic features, functions and physiological regulation. In many ways, collateral vessels are as different from general and lymphatic vessels, as the latter are from each other. So much so that collaterals might be regarded as comprising a "third" circulation – the *Collateral* circulation.

How so? Most healthy adult tissues have a population of native (pre-existing) collaterals that cross-connect one or more distal-most arterioles of one arterial tree with those of an opposing arterial tree. There are also large, anatomically named collateral conduit arteries in certain regions of the body (eg, the *recurrent radial artery* around the elbow). This chapter will focus on the microvascular collaterals. These have a much more varied abundance and importance in coronary artery disease (CAD), peripheral artery disease (PAD) and intracranial thromboembolic stroke. Native collaterals average 10-40 microns in diameter in healthy tissues. They are relatively rare. For example, the thigh of the mouse is estimated to have 10-20 of these vessels [1], while similarly sized arterioles in the thigh's general circulation number in the thousands. Native collaterals are unique in four major ways. By inter-connecting arterioles from adjacent trees, the process by which they form somehow defies instructions from canonical patterning molecules (eg, ephrin-B2 and eph-B4) that in the general circulation insure that arterioles connect to capillaries that, in turn, connect to venules. Perhaps more remarkable—owing to absence of a consistent pressure drop across them—blood flow in collaterals in healthy tissues is exceedingly low and disturbed or oscillatory [2], conditions that in the general circulation promote vascular wall inflammation, hemostasis and pruning. Yet native collaterals persist in these conditions of low shear stress and high circumferential wall-stress.

A third unique feature of collaterals is that they undergo extensive growth in the radial and longitudinal directions over a period of days-to-weeks, when unidirectional shear-stress is induced by obstruction of flow into one of the trees they cross-connect. This growth response, termed "arteriogenesis" by Schaper and colleagues [3], increases diameter up to 10-fold. This greatly increases collateral conductance and thus the protective function of these vessels as "endogenous bypass vessels" to restore perfusion in the dependent tissue towards resting values. However, the extent (ie, density and diameter) of these vessels in a healthy tissue determines not only the blood flow and therefore severity of ischemic tissue injury immediately after acute thromboembolic obstruction, but also the number of collaterals that are then able to undergo arteriogenesis over time. A fourth special feature is that the extent of the native collateral circulation varies dramatically among healthy individuals as a function of not only genetic background [4,5] but also from environmental factors (see below). Yet, the general and lymphatic circulations in such individuals are comparable in both form and function.

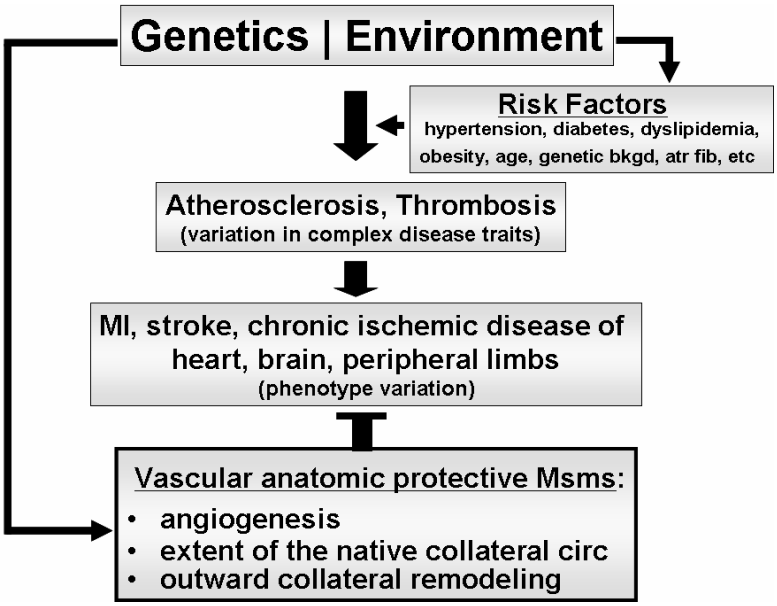

Figure 1: Individual variation in genetic background and environmental factors accounts for variation in risk factors and complex disease traits, which in turn are responsible for phenotypic variation in vascular diseases. As well, variation in protective mechanisms, including extent of the native collateral circulation and its capacity to remodel in obstructive disease, can be ascribed to individual differences in genetic and environmental factors.

Despite these special features, until recently almost nothing was known about when or how this circulation forms, how these vessels are able to persist in such disturbed hemodynamic conditions, or the basis for the wide variation in extent of this circulation among otherwise healthy individuals. As with other phenotypic differences, variation in collateral extent is attributable to genetic and/or environmental factors (Fig. **1**). However, the nature of these factors has, until recently, been unknown. This chapter will summarize recent studies that have begun to address these questions. Mechanisms of collateral remodeling in obstructive disease—discussed in subsequent chapters and recent reviews—will be limited to summarizing evidence identifying genetic factors that impact this process.

Embryonic and early postnatal formation of the native collateral circulation

It is estimated that one-fifth of patients with CAD are not candidates for percutaneous coronary intervention or coronary artery bypass grafting [6]. Hence, arteriogenic therapies to boost positive remodeling of exiting collaterals, or collaterogenic therapies to induce formation of additional ones, have been pursued for more than a decade. However, no drug has emerged for clinical use with either benefit, owing to lack of efficacy, co-morbidities or unacceptable side effects [7]. Understanding how native collaterals naturally form could provide important clues about how to induce more to form when obstructive disease strikes. Unfortunately, there are major outstanding questions about this process, although they are now beginning to be addressed.

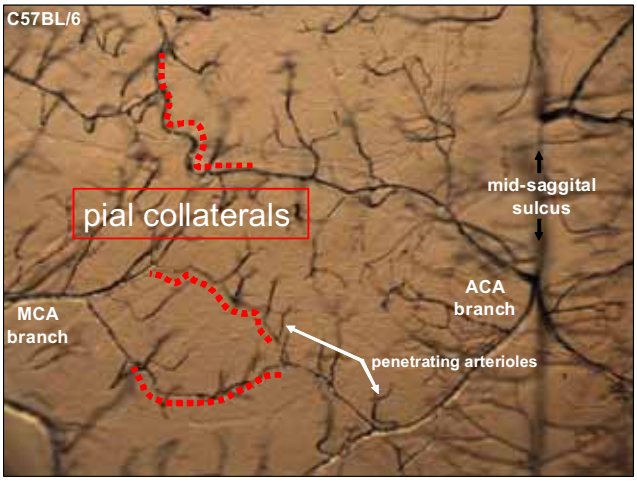

Figure 2: Native pial collaterals (denoted by red dotted lines) interconnecting distal arterioles of the middle cerebral and anterior cerebral artery trees in an adult mouse. The vasculature was dilated, fixed and filled with a high-viscosity material that does not cross capillaries, followed by clearance of tissue refractance, to highlight penetrating arterioles (two are denoted) and confirm that native collaterals are confined to the pial surface. From Ref 16.

When and how do native collaterals form and grow to their mature density, diameter, length and characteristic tortuosity in healthy tissues? In the embryo, neonate or during growth to adulthood? Surprisingly, very little has been reported, although several possibilities have been proposed [8]. What information we have comes from early studies examining the cerebral circulation, where anastomoses were noted to already be present in newborn mice [9] and rats [10] cross-connecting some of the distal-most branches of the anterior, middle and posterior cerebral artery (ACA, MCA, PCA) trees. Such collaterals, which range from 10-40 microns in diameter, are present in all adult mammalian species examined (including humans) [11], as well as in birds and reptiles [12,13], and in most tissue types. We have recently begun to address the above questions in murine brain and several other tissues. The cerebral cortex is particularly well suited because, as Figure **2** shows in an adult mouse, its collateral circulation resides outside of the brain in the pia mater where it can be imaged with high fidelity after craniotomy, dilation, fixation and staining or filling with casting or contrast material [4,14-16]. Penetrating arterioles branch from both pial arteries and collaterals into the cortex, where they supply intracerebral terminal arterioles ("end arteries"), capillaries and post-capillary venules [16].

Studies in newborn mice have confirmed that pial collaterals are already present by postnatal day-1 (P1) [15,16]. We have also found that "gene-dose-dependent" reduction or increase in expression of either *Vegfa* or *Clic4* (Chloride Intracellular Channel-4) reduces or

increases collateral number and diameter at P1, respectively (Fig. **3**) [15,16]. Altered expression of these proteins also modulates an obligate postnatal pruning process that reduces the collateral density by the end of the third postnatal week to the density present in the adult (Fig. **3**). Experimentally targeted changes in collateral abundance significantly impact the severity of stroke after MCA occlusion (Fig. **3**) [15,16]. Thus, the collateral circulation is established sometime during embryogenesis and subsequently undergoes early pruning, with the above two genes involved in both processes.

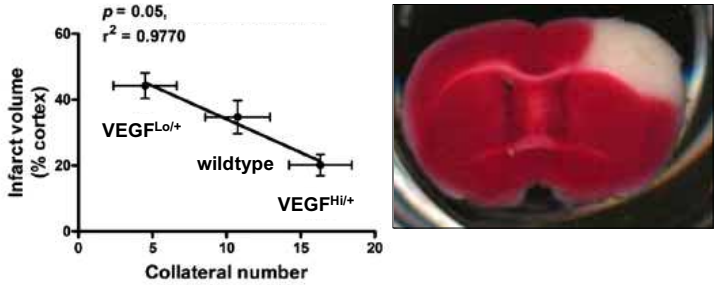

Figure 3: The number of native pial collaterals interconnecting the MCA and PCA trees (per cerebral hemisphere) in healthy adult mice is affected by VEGF-A expression. Mice are hypo- or hypermorphic for VEGF-A expression at one allele. Infarct volume (white region), measured 24h after permanent unilateral MCA occlusion, correlates closely with collateral number. From Ref 15. Comparable findings were obtained in intact hindlimb and after femoral artery ligation (Ref 4), and also in both brain and hindlimb for CLIC4 expression (Ref 16).

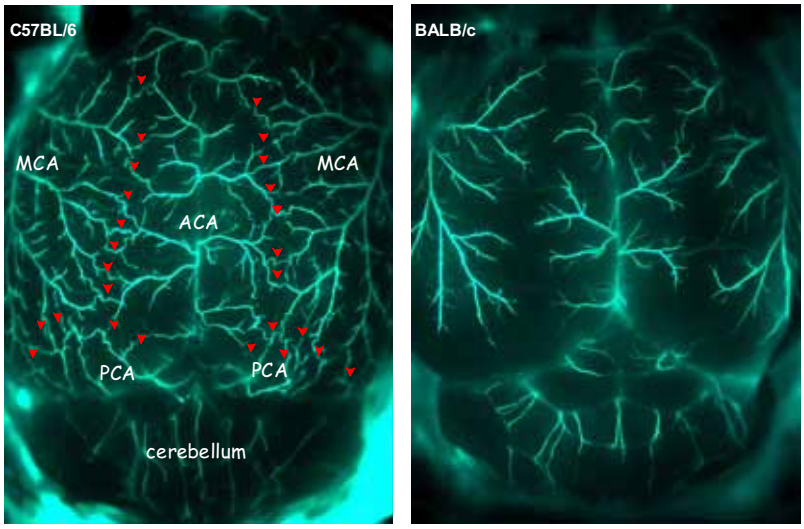

Figure 4: Wide difference in native pial collateral density (red stars) between MCA, ACA and PCA in two inbred mouse strains. BALB/c have almost no collaterals and suffer massive strokes after MCAO, compared to C57BL/6 where they are small and cause little or no functional impairment. The vasculature was dilated, fixed and filled with a high-viscosity fluorescent material unable to cross capillaries. Collateral density in skeletal muscle and intestine in the two strains shows a similar but less dramatic difference. From Ref 14.

But *when* in the embryo do collaterals form? What is the nature of the postnatal maturation phase? What are the responsible mechanisms and does genetic background affect them? We [2] focused on two strains of mice, C57BL/6 and BALB/c, with large differences in adult collateral density in multiple tissues (Fig. **4**) [2,4,14,17]. In fact, these strains have the largest difference among more than 15 adult inbred strains examined [4]. We reasoned that comparing collaterogenesis in such divergent strains may begin to answer the above questions. In both strains, collaterals appear by ~E14.5-15.5 as a plexus of ring-like ephrin-B2-positive endothelial tubes interconnecting the crowns of the cerebral artery trees (Fig. **5A,B**) [2]. This "primary collateral plexus" appears abruptly after the cerebral artery trees have become well-developed. Collateral density increases during expansion of the plexus through ~E18.5 (Fig. **5C**).

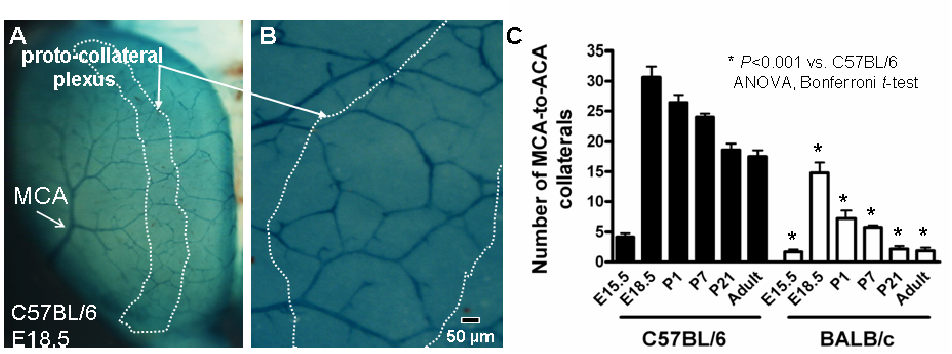

Figure 5: A,B, Collaterogenesis in the mouse cerebral circulation. Collaterals begin forming at ~E15.5 as a proto-collateral plexus of ephrin-B2-positive endothelial cell tubes in the region between the already-formed cerebral artery trees that expands through E18.5. BALB/c mice form fewer collaterals during embryogenesis. The nascent collateral circulation of BALB/c mice undergoes greater postnatal pruning. From Ref 2.

However, many fewer proto-collaterals form in the BALB/c strain. This is not due to the presence of fewer endothelial cells, ie, the area of the primary embryonic capillary plexus present at ~E9.0 and E12.5 in the region between the cerebral artery trees that will become the "collateral zone" is similar in both strains [2]. Nor is it from differences in brain size or branching morphogenesis of the cerebral artery trees [2]. The nascent collaterals of BALB/c mice also undergoe more pruning, which occurs from E18.5 through the first few weeks after birth, ie, the collateral maturation phase (Fig. **5C**). Collaterals that are retained during this phase increase their diameter and length and begin to acquire tortuosity – a characteristic of collaterals. However, BALB/c collaterals are smaller in diameter at birth and grow less in both length and diameter during maturation, features in common with mice deficient in VEGF-A or CLIC4 expression [15,16]. These differences are also not due to differences in mural cells. Pericyte density on nascent collaterals is comparable in both strains [2], and acquisition of SMCs does not occur until after P7 (Fig. **6A-C**).

So if not from the above mechanisms, what is responsible for formation of fewer, smaller diameter collaterals in the BALB/c embryo and less growth of those that do form in the neonate? *In silico* analysis to test for the presence of expression quantitative trait loci (eQTL), using a database obtained from a recombinant inbred set of mice derived from C57BL/6 and BALB/c parentals, suggested the presence of a polymorphism at or near the *Vegfa* locus linking low VEGF-A expression (a feature of the BALB/c strain) to predicted low PDGF-B and high angiopoietin-2 expression, compared to C57BL/6 mice [14]. We recently confirmed these differences in baseline expression in tissue samples taken from the collateral zone between the MCA and ACA trees of perinatal mice (Fig. **6D**) [2]. Adult BALB/c mice also express less VEGF-A during another time of vascular growth—during ischemia/hypoxia in skeletal muscle, brain and pulmonary airways [14,15,18,19]. Mutant mice haplo-insufficient for VEGF-R1 (Flt1) also form fewer collaterals in the embryo

(unpublished). These studies suggest that VEGF-A, VEGF-R1, angiopoietin-2, PDGF-B and CLIC4 are important in formation and maturation of the proto-collateral plexus in the embryo and neonate and thus in determining the extent of the collateral circulation in the adult. Additional studies are needed to define *how* the above factors, which are key regulatory molecules in branching morphogenesis, muralization and pruning of the general circulation [20-23], impact these processes in collateral formation, as well as to identify other candidate molecules, localize their expression, and alter their activity for effect on collateral formation and maturation. As well, whether common mechanisms control collaterogenesis in different tissues is not known, although qualitatively similar strain-specific differences in density have been found in skeletal muscle, brain and intestine of several adult inbred and mutant mouse strains [14-16]. Collaterals in mouse skeletal muscle are well-developed at birth (Fig. **7**), indicating that their formation in tissues in addition to brain also occurs during embryogenesis.

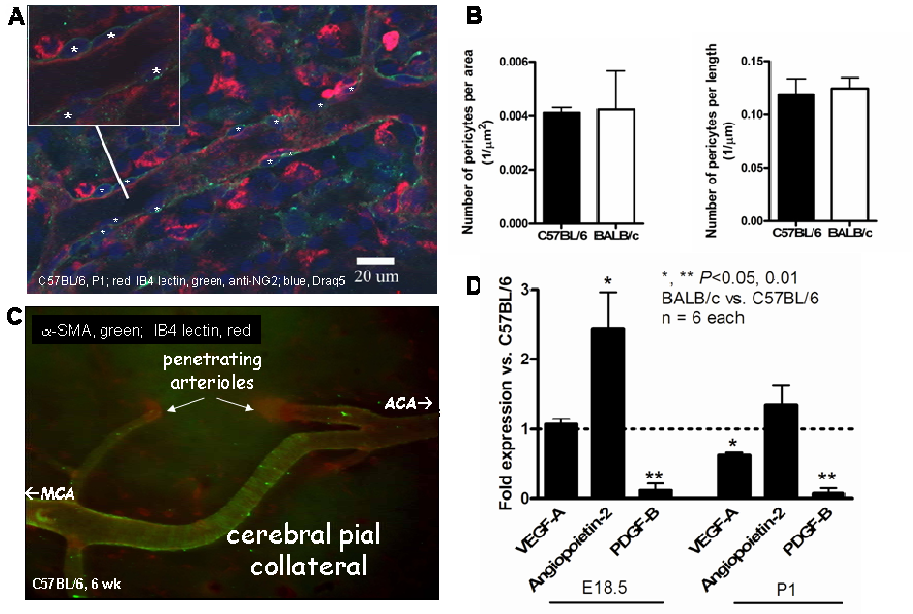

Figure 6: A, Confocal image of isolectin-B4 (red), anti-NG2 (green) and Draq5 (blue); stars, pericytes. B, Number of pericytes per collateral length-x-width and per length are not different between strains on postnatal day1 (P1, shown) (or E15.5 (data not shown)). C, Collaterals do not acquire smooth muscle cell coverage until after P7 (H Zhang, unpublished). D, qPCR of tissue samples taken from the collateral zone between MCA and ACA. BALB/c collaterals have increased angiopoietin-2, reduced PDGF-B and reduced VEGF-A expression. Data normalized to 18S rRNA. Data in A, B and D from Ref 2.

VEGF-A is a determinant of collateral formation in the embryo

Given the above data suggesting that VEGF-A levels influence collateral formation in the embryo [15], we have begun to further investigate its involvement using mouse embryos constitutively over- and under-expressing global endogenous VEGF-A [24,25]. Collaterals between the MCA and ACA trees first become evident shortly after emergence of the ACA tree from the mid-saggital sulcus at E14.5, with the number that form being greater in over-expressing and smaller in under-expressing embryos (Fig. **8A**). The vessels first appear as thin extensions (Fig. **8B,** arrows), often at points where penetrating arterioles that descend into the cortex. These extensions appear to arise from sprouting of endothelial tip cells. Interestingly, these extensions span the opposing arterial trees by forming on top of the existing pial capillary plexus (Fig. **8B**). This is consistent with formation of the primary

embryonic collateral plexus well after formation of the primary embryonic capillary plexus [2]. This is also different from how the pial arterial trees form, which involves remodeling of the primary embryonic capillary plexus. Moreover, the collateral plexus forms as an artery-fated plexus of endothelial cell tubes expressing ephrin-B2 [2]. Current studies are using inducible mice to pinpoint the cell source and time-frame over which VEGF acts to modulate collateral formation. No doubt, the above process likely has some features that are specific for the special anatomy of the pial circulation. However as mentioned above, evidence suggests common signaling molecules and genetic polymorphisms control collaterogenesis in different tissue types despite their unique anatomic arrangements of the general circulation.

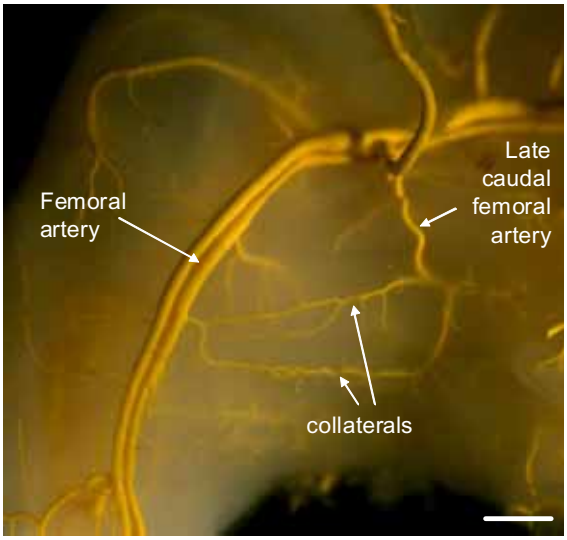

Figure 7: Collaterals in the adductor musculature form during embryogenesis and are well developed at postnatal day 1. Scale bar=500μm.

Intriguing results from Vogel, Marti *et al.* [26-28] support the concept that VEGF expression regulates collateral formation. These investigators [27] designed a transgenic mouse with increased expression of VEGF driven by a neuron-specific promoter to investigate effects on cerebral vascular function. Total vascular volume increased, as determined by micro-CT, but maximal cerebral blood flow was only minimally increased. The authors proposed an increase in formation of arteriole-to-arteriole "anastomoses" to account for the findings, although segmental analysis could not distinguish such structures for confirmation [28]. Another study found that these mice experienced smaller infarct volumes and neurological deficits after MCA occlusion, plus an increase in blood flow in the ACA distribution [26]. These findings are consistent with an increase in ACA-supplied collateral-dependent flow to the outer penumbral region of the occluded MCA tree. This would be expected if pial collateral density and/or diameter are increased.

Once nascent collateral connections form in the embryo, how are they maintained? Platelet-derived growth factor (PDGF)-B promotes the migration of mesenchymal cells to newly formed vessels, where they differentiate into pericytes that stabilize the endothelial network [29,30]. Price *et al.* [20] examined the progression of coverage of the developing vasculature by cells expressing smooth muscle actin in the early postnatal rat gracilis. They found that coverage of arteriole arcades (anastomoses; possibly in the process of formation) by cell processes that express smooth muscle actin, was continuous on the opposing arterioles but sparsely distributed on the capillary-like arcade segment. This observation is consistent with the hypothesis that collaterals form from the fusion of capillary-like

branches sprouting from established arterioles rather than forming *de novo*. This concept is also congruent with our findings that proto-collaterals express the arterial endothelial cell marker, ephrin-B2 [2]. Why collaterals form only between certain distal arterioles is not known but may be related to small localized differences in oxygen tension and VEGF gradients or hemodynamic forces. The latter would be highly disturbed when sprouting nascent collaterals first cross-connect opposing arterial trees and experience high pressure from both ends. In this regard, both increased shear stress and wall stress can increase VEGF-A expression in vascular tissue [31-33]. Interestingly, as discussed above, BALB/c embryos, which form many fewer collaterals than C57BL/6, have sharply reduced expression of PDGF-B in the pial collateral zone at E18.5 [2]. These findings support the idea that PDGF-B expression during embryogenesis is necessary to recruit pericytes to newly formed collaterals and insure their persistence.

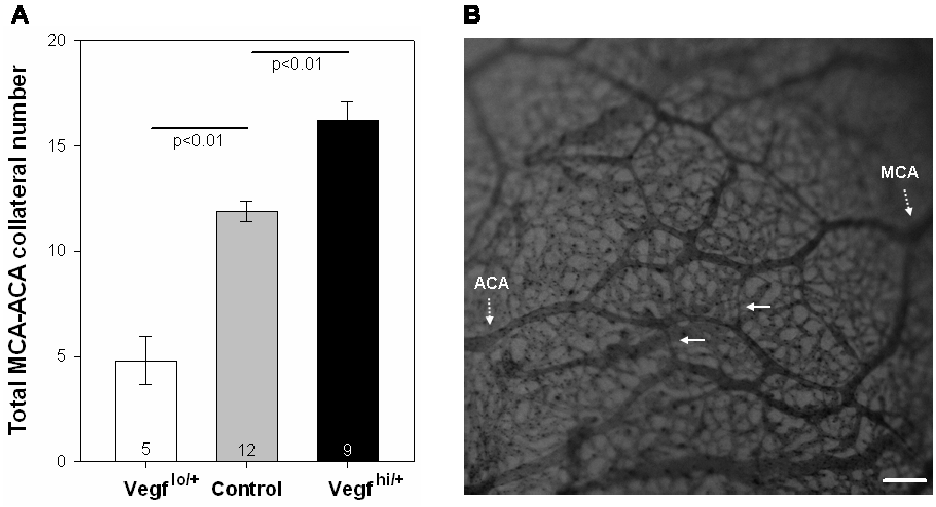

Figure 8: A, VEGF-A expression dictates pial collateral density at embryonic day (E)16.5. Embryos underexpressing VEGF (Vegf lo/+) form fewer collaterals at this developmental stage than wildtype littermates. Conversely, embryos overexpressing VEGF (Vegf hi/+) form significantly more collaterals. B, Collateral vessels are forming by ~E14.5 and often appear as narrow endothelial cords situated superficial to the existing pial capillary plexus.

Based on the above findings, a model of cerebral collateral formation during embryogenesis is beginning to emerge (Fig**. 9**). CLIC4 deficient mice form fewer collaterals [16], and CLIC4 is known to promote endothelial proliferation, lumenization and network formation [34,35]. Shukla *et al.* showed in vascular smooth muscles cells that TGF-β signaling stimulates cytoplasmic CLIC4 to associate with the transcription factor Schnurri-2 [36], plus activated Smads and β-catenin, leading to nuclear translocation and induction of VEGF-A expression [37]. VEGF has an essential role in vessel formation, branching and guidance. The longer splice isoforms (VEGF164 and VEGF188) contain basic amino acids that interact with negatively charged heparin sulfate side chains on cell surfaces and extracellular matrix molecules, serving to anchor VEGF near the secreting cell. Endothelial cells respond to this gradient via Flk1 (VEGFR2) and initiate/interact with multiple intracellular signaling cascades (see [38-40] for recent reviews) that modulate cell activity and phenotype. During angiogenesis, VEGF signaling in tandem with notch/delta signaling lead to the "selection" of an endothelial tip cell that undergoes cellular changes and extends filopodia toward the VEGF source. Neighboring cells are signaled to become stalk cells, trailing behind the mobile tip cell. Together they extend a cord that becomes a branch of the existing vessel. The local concentration of VEGF available to bind to Flk1 is modulated by the presence of Flt1 (VEGFR1), which in turn regulates endothelial branching [41,42]. Flt1 has high affinity for VEGF and is believed to act as a VEGF sink, reducing the amount of

VEGF available to bind to Flk1 and initiate kinase activity. This cord of cells extends and joins with a similar cord, or to an arterial branch. The cord then develops a lumen, becomes pressurized by blood and forms a proto-collateral. This simplified model of collaterogenesis can be expected to undergo much refinement as future work unfolds.

Extent of the collateral circulation varies widely among individuals

In the late 19th century various mammalian species were noted to have differences in both the presence of inter-coronary artery collaterals and the amount of tissue injury after ligation of one coronary artery [43]. Schaper [44] was the first to systematically compare infarct size, risk zone and collateral-dependent flow. Rat, rabbit, pig, sheep, pony, baboon and wild boar sustained large transmural infarctions after coronary ligation, in association with low collateral flow, while dog and cat experienced more slowly developing and incomplete infarctions associated with greater collateral flow, and guinea pig sustained little or no infarction and had collateral flows approximating those present before ligation. These findings were confirmed by Maxwell *et al.* [45]. Large variations in collaterals and ischemic lesion size have also been described in humans with regard to the presence and diameter of the ophthalmic artery collaterals and the bilateral anterior and posterior communicating arteries of the circle of Willis (the so-called "primary" cerebral collaterals) [46,47]. Similar variability in these primary collaterals has been described in experimental animals, including inbred mouse strains [48].

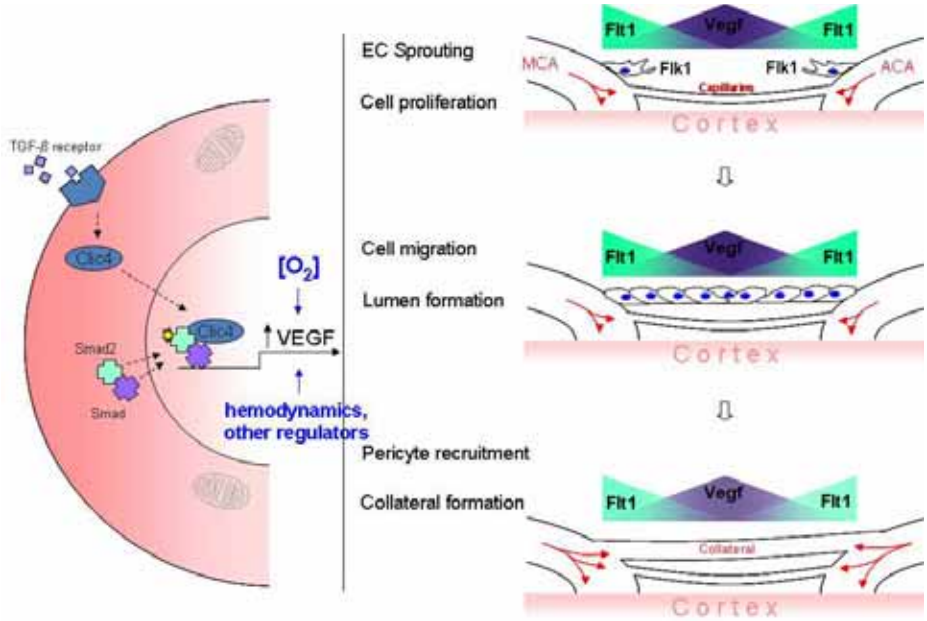

Figure 9: Model depicting possible mechanisms of collaterogenesis in the cerebral circulation of the embryo. TGF-β, CLIC4 and other factors increase VEGF expression. VEGF isoforms and Flt1 create a local VEGF gradient which causes endothelial cells to express a tip cell phenotype, extend filopodia and mobilize up the gradient. Neighboring endothelial cells receive tip cell signals that suppress the tip cell phenotype and initiate a stalk cell phenotype. This proto-collateral plexus extends over the pial capillary plexus and joins other chords emanating from opposing arterioles, followed by lumen formation and cross-connection of the arterial trees. See text for additional details.

There is also recent evidence of significant variation in the pial collateral circulation in humans (discussed below). And large variation in clinical outcomes of patients with PAD suggests significant variation in collaterals to the lower extremities. However, due to the small size of most collaterals, an analysis of the native collateral circulation in the extremities of healthy individuals has not been conducted. Thus, it is not known whether this variation reflects differences in collateral extent before disease, or differences in remodeling after it develops.

The severity of tissue injury and functional impairment in patients with CAD, PAD and in individuals suffering acute thromboembolic stroke are known to vary widely. Although much effort has been directed at identifying the potential sources of this variation, the possibility that individual differences in the native collateral circulation exist and contribute significantly has received little attention. Findings from several recent studies, however, support this hypothesis. In an important recent paper, Meier, Seiler and colleagues [5] reported data collected over ten years from 106 *healthy* human subjects showing that pressure-derived coronary collateral flow index (CFI_p), an index of conductance of the collateral circulation, varied by 10-fold. This variation is remarkable, since these individuals were free of known disease. Although CFI_p may be affected by other factors [49], this suggests that significant variation exists in extent of the *native* coronary collateral circulation in humans. That is, the variation they measured could not result from differences in collateral remodeling, which is known from animal studies to vary depending on degree and duration of obstruction [50-57]. In contrast, other studies—whether using CFI_p or other measures (eg, angiography)—examined patients with CAD or PAD, wherein differences in collateral remodeling confound any interpretation *vis-à-vis* extent of the native collateral circulation.

Additional findings in patients with stroke support the concept that collateral extent varies in healthy human tissues. Building on earlier studies [eg, 58], Bang *et al.* [59] and others [60-62] used dynamic angiography in patients suffering acute proximal obstruction of the MCA and found that retrograde perfusion of the MCA tree downstream from the occlusion, which is dependent on collateral extent between the MCA, ACA and PCA trees, varies substantially. Since obstruction is sudden in thromboembolic stroke, individual differences in collateral remodeling are eliminated as a complication. However, variation in other factors, such as downstream stagnant thrombosis, thrombolysis and arterial pressure, can affect collateral-dependent retrograde perfusion. Further support for significant variation in collateral abundance is provided by the well known observation that residual flow to the extremities shows large differences after acute trauma-induced interruption of a major proximal artery in otherwise healthy individuals [63].

Natural genetic variation contributes to variation in the native collateral circulation

The above differences could be due, in part, to polymorphisms in genes controlling the formation and maturation phases of collaterogenesis. Until recently [2,4,14-16], nothing was known about this possibility. Zhang and coworkers [4] quantified native pial collateral dimensions in 15 inbred mouse strains. Remarkably, pial collateral density differed by 15-fold (Fig. **10**). They then correlated this and other parameters with infarct volumes measured 24h after permanent MCA occlusion, the latter reported by Keum and Marchuk for the same strains [64]. A Poiseuille-like equation—incorporating collateral number, diameter, length, hematocrit and MCA territory among the strains—closely predicted variation in infarct volume [4]. These investigators also measured increased collateral lumen diameter (remodeling) induced by permanent MCA occlusion, and observed wide differences among the strains [4]. Interestingly, the variation did not follow the strain-specific pattern for native number and diameter shown in Figure **10**. This suggests that different signaling mechanisms are responsible for collateral formation versus remodeling (confirmed below). The finding of such large natural genetic variation in the collateral circulation is surprising, since the general and lymphatic circulations appear to be comparable in these strains. This work also provided a mouse phenotype database useful for future work aimed not only at studying stroke, but also for identifying the genetic loci underlying this wide collateral variation, including optimal strains to cross for QTL and haplotype mapping to identify candidate genes regulating collaterogenesis and remodeling.

As discussed above, variation in coronary collateral abundance has been described among non-murine mammalian species [44,45]. Since those species examined were mostly inbred or domesticated, it is not clear if the differences reflect true variation among species, or if other inbred strains within a given species would exhibit the range of variation seen in mice [4] and, potentially, humans [5]. In this latter study in which 739 patients with stable CAD were followed over a 10-year interval, individuals with good collateral flow index ($CFI_p \geq 0.25$) had a 75% decrease in mortality, compared to those with poor CFI_p values. However, as in previous studies examining patients with ischemic disease, it is not possible to differentiate the contribution of variation in native extent versus collateral remodeling. Elsewhere, in patients with acute thromboembolic MCA stroke, those with angiographic evidence of good pial collateral flow had better functional recovery and less incidence of hemorrhage following thrombolytic therapy [58-62]. These benefits were attributed to less ischemic destruction of brain tissue and blood vessel walls, secondary to better collateral-dependent retrograde perfusion of the MCA tree. However, since pial collaterals could not be directly imaged, whether differences in retrograde perfusion in stroke patients reflect differences in native collateral extent versus other mechanisms awaits advances in imaging.

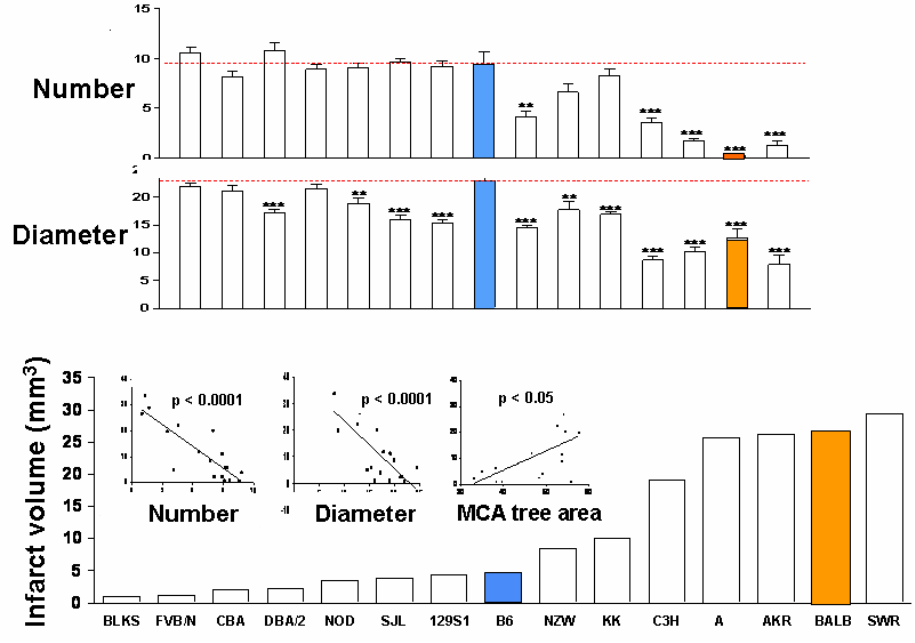

Figure 10: Wide variation in infarct volume 24h after permanent unilateral MCA occlusion is highly correlated with wide variation in collateral number and diameter (per hemisphere), and less with MCA tree territory, among 15 inbred mouse strains (stain names arranged in same order in all 3 bar graphs). B6, C57BL/6. From Ref 4. Infarct volume from Ref 64.

Genetic loci linked to variation in the native collateral circulation and severity of ischemic tissue injury

Besides using the candidate gene approach, which showed that variation in expression of VEGF-A and CLIC4 contributes to variation in collaterogenesis [15,16], we recently undertook an unbiased approach to define the underlying genetic architecture and regulatory loci governing variation in collateral extent [65]. Pial collateral number and diameter were determined in 221 progeny of a C57BL/6 x BALB/c reciprocal intercross, and subjected to genome-wide mapping to identify QTL governing the large difference in these traits in the parental strains. Four highly significant QTL were identified on chromosomes 1, 3, 7 and 8, including epistasis between the first two loci. The loci together account for 70% of the 15-

fold variation in native collateral number present in the F2 population (the same variation seen in the parentals). The most significant QTL on chromosome 7 (accounting for 37% of the variation in number) was also highly linked to variation in native collateral diameter (no other QTL for diameter were found). Chromosome substitution strain (CSS) analysis, using lines from the A/J→C57BL/6 strain set, confirmed the functional (causal) importance of this locus. Association mapping using the above-mentioned 15-strain database pinpointed a significant (p=0.00002) region within the chromosome 7 QTL harboring several candidate genes that are currently under investigation. Interestingly, variation in collateral remodeling after permanent MCA occlusion was linked to a QTL on chromosome 11. No differences in native collateral extent or remodeling were evident as a function of sex or parental genotype.

Thus, collateral number, diameter and collateral remodeling are heritable complex traits, with one QTL on chromosome 7 accounting for the majority of the variation in number and diameter between the C57BL/6 and BALB/c strains [65]. Interestingly, using identical F2 crosses, the same QTL on chromosome 7 was linked to necrosis score and recovery of blood flow after femoral artery ligation [66] and to infarct volume 24h after permanent MCA occlusion [63]. CSS analysis confirmed causality in both studies. Our findings suggest that this locus has a central role in collaterogenesis, and through this effect, constitutes a major physiological mechanism underlying these previously reported QTL. Recently, Meier, Seiler and colleagues [67] examined gene expression in 50 patients stratified for poor versus well developed collateral flow (CFI_p < vs. ≥ 0.21, respectively—the latter with no angiographically significant CAD). Four genes, ACTN1, EGFL7, LIMS1 and RPS6KA3, were differentially expressed, consistent with the concept that genetic factors may play a role in determining the abundance of native coronary collaterals in healthy individuals. Interestingly, EGFL7 is a stalk cell protein. Zhang, Doevendans *et al.* [68] genotyped 226 CAD patients, stratified for good versus poor collaterals, at SNPs in 54 genes potentially involved in collateral remodeling, identifying 9 that may be linked to the abundance of angiographically visible collaterals. These interesting studies identify genes that may be involved in variation in formation of the collateral circulation or its remodeling in obstructive disease.

Environmental factors cause variation in the native collateral circulation: Collateral rarefaction

The mouse studies discussed above identify several signaling molecules important in collateral formation and show that natural variation in specific genes or regulatory loci are causally involved. What about "environmental" (as opposed to "genetic") factors? Are there conditions, risk factors or diseases that cause loss of collaterals, narrowing of their diameter or an increase in their length/tortuosity? Are there environmental factors that have opposite effects, providing protection should occlusive disease develop? Like most of the topics addressed in this chapter, little is known. This is, in part, because studies examining collateral function in disease or in the presence of risk factors have not examined native collateral extent—which is not easily measured. Indeed as discussed in subsequent chapters, several decades of research have identified a number of diseases and risk factors that alter (mostly impair) collateral remodeling in arterial obstructive settings. Nevertheless, recent studies are beginning to examine these questions. Although collateral density and diameter in young adult mice (3 months age) are largely specified within the first 3 postnatal weeks (Fig. **5C**) [2,14-16], collaterals—at least in the brain—continue to increase in length (ie, tortuosity) during growth to adulthood [2]. This change, plus the early postnatal phase of collateral maturation [2] (eg, Fig. **5C**), suggests that environmental factors present after birth could impact the extent of the collateral circulation in adults.

One unavoidable risk factor strongly influenced by environment is natural aging (although aging also evidences significant genetic variation). It is well known that aging is associated with poor collateral remodeling in obstructive disease [69-71]. Aging may also cause arteriolar [72,73] and capillary rarefaction in certain tissues [74,75]. Our group together with S. Epstein and colleagues recently examined whether aging affects the native collateral circulation [unpublished results]. Collateral density and diameter declined progressively among 3, 16, 24 and 31 month-old mice, and collateral tortuosity increased. These changes

were associated with a decrease in conductance of the pial collateral circulation, eg, 8-fold in the 24 month-old group. Furthermore, this collateral rarefaction occurred in both hindlimb and brain, in association with "age-dose-dependent" increases in ischemia and tissue injury after femoral artery and MCA ligation. In support, an earlier study noted fewer pial arteriolar anastomoses in aged rats [73]. While the mechanism for these changes has not yet been determined, age-associated rarefaction of arterioles has been linked to reduced eNOS/NO activity/bioavailability [Epstein *et al.*, unpublished]. Furthermore, although eNOS (and eNOS/iNOS) deficient mice have no difference in collateral extent at birth or 3 weeks later in hindlimb or brain—indicating that eNOS/iNOS is not required for collateral formation or maturation—collateral density declines by 3 months and more by 6 months of age in both tissues [77]. These data show that eNOS is required for maintenance of the native collateral circulation. They also suggest that aging, "life-style" factors, and pathologic conditions that induce endothelial dysfunction could cause collateral circulatory insufficiency before obstructive disease develops.

Environmental factors that increase extent of the native collateral circulation

Neocollateral formation. Can native collateral density or diameter be increased by pathologic or other environmental conditions? Could preventative physiologic or therapeutic strategies be developed to achieve this before or after onset of obstructive disease? Other chapters in this volume address the mechanisms whereby increased flow and shear stress imposed by arterial obstruction initiate collateral remodeling. However, whether new collaterals ("neocollaterals") can form in adult tissue is a long-standing controversial question. If genetic background can limit the extent of the collateral circulation in healthy tissues, understanding if it is possible—and how—to induce formation of neocollaterals in obstructive disease are critical questions that need to be answered in order to enable development of collaterogenic therapies.

It has been difficult to determine if the appearance of more collaterals in experimental or clinical studies of artery obstruction in heart or peripheral limbs are from formation of neocollaterals or from shear-dependent positive remodeling of collaterals that were too small to be detected before obstruction. Counting smooth muscle actin-positive vessels in tissue sections taken from the region between the crowns of artery trees, given their 3-dimensional distribution deep in most tissues, also has limitations. Location of their collateral zones is needed to prevent counting distal arterioles within the trees themselves. This is further complicated by the fact that chronic dilation and increased flow within an arterial tree stimulate proliferation and migration of smooth muscle cells onto the arterial end of the capillaries [77-79]. These muralized capillaries then enlarge their diameters and lengthen the terminal arteriole end of the tree—a process termed distal muscularization or arterialization [77]. Chronic dilation and increased flow in the arterial feed vessels upstream of a collateral are also caused by the drop in resistance and pressure below the collateral when the trunk of the arterial tree downstream from it becomes obstructed. Predicted flow-mediated dilation of the upstream arterial feed vessels could also contribute to this. We have recently confirmed that an immediate and sustained dilation and increased flow occur in the arteries immediately upstream of hindlimb collaterals after femoral artery ligation. This provides a significant "feed-forward" component of collateral perfusion to the at-risk limb [76]. Prolonged upstream dilation may also promote outward remodeling of the feed arteries [80]. Clearly, better understanding of the environmental (and genetic) factors that control and modulate collateral outward remodeling in healthy and obstructed tissues is needed.

Distal muscularization alone cannot form a neocollateral between two opposing trees when one is occluded. Formation requires muscularization across the length (ie, hundreds of microns) of a pre-existing or newly formed capillary. The latter can occur from ischemic sprouting or intussusception, followed by lumen expansion, muralization and wall thickening. Pruning of connections to venules is also required. Price and Skalak used theoretical modeling to show how pruning in a collateral circuit could occur [81]. And experimental studies have confirmed that elevated shear stress across obtuse branch angles caused by reversed flow can induce pruning [82-85]. Although native collaterals are more easily quantified in the brain than in other tissues, whether neocollaterals form after

permanent MCA occlusion is nevertheless also controversial [86,87]. We have recently found supportive evidence [4]: Among 15 inbred mouse strains, 4 strains formed neocollaterals after MCA occlusion, and these were strains that had particularly low pre-existing collateral densities (including BALB/c). This genetic variability in the capacity to form neocollaterals could help explain some of the previous disagreements in the literature. In addition, capillaries in the pia mater are lost after birth. Thus, it may be particularly difficult to form neocollaterals after stroke, compared to other tissues. Mac Gabhann and Price recently demonstrated in an elegant study that neocollaterals form in the mouse spinotrapezius muscle after arterial ligation [109]. Recovery of perfusion in BALB/c mice was associated with arterialization of capillaries interconnecting the ligated and intact arterial trees, followed by remodeling of their diameter to twice normal. These findings [4] begin to address a long-standing question that has clinical significance and provide model systems to study basic mechanisms responsible for—and possible therapeutic approaches to—induce neocollateral formation.

The intriguing A-V fistula hindlimb model [80] may provide a model to investigate shear stress as a potential stimulus for neocollateral formation. In addition, two interesting embryo ligation models have been introduced. le Noble *et al.* [88, see also Chapter 2] showed in the early embryonic chick yolk sac that ligation of the right vitelline artery caused the distal venous plexus that it flows into to reverse flow, express arterial endothelial cell markers and remodel into an extension of the left vitelline artery tree into the right side of the yolk sac. There it drained into the distal right vitelline artery tree which, experiencing reversed flow, remodeled into a venous tree expressing venous endothelial cell markers. Gray *et al.* [89] showed something similar in the zebrafish embryo. Ligation of the aorta at its midpoint induced remodeling of intestinal inter-connections to form a collateral circuit back to the aorta distal to the ligation, resulting in little or no disturbance in tail growth. Defining the molecular mechanisms guiding formation of these collateral-like networks, which in these models involve ligation-induced co-opting of venous plexi in the highly plastic embryonic vasculature, may provide insight into how neocollaterals might form in the adult. Whether neocollateral formation in occlusive disease is driven by ischemic and/or hemodynamic signals is an important unanswered question. In addition, understanding the molecular-genetic mechanisms governing formation of the collateral circulation in the embryo may provide clues regarding the signaling molecules responsible for formation of neocollaterals in the adult.

Does chronic "exercising of collaterals" increase extent of the native collateral circulation?

Many reports have shown that exercise training has a beneficial effect in patients with CAD [90,91] and PAD [92] and in animal models of arterial occlusion [93]. The latter have identified several mechanisms, including enhanced collateral remodeling, improved flow-mediated dilation of arterial beds upstream of the collateral network, and lower resistance below the collaterals. However, Yang and colleagues found no evidence that exercise training increases collateral extent [94]. Six weeks of treadmill training of adult rats augmented collateral-dependent flow by 70%, when measured after acute femoral artery ligation at the end of the training period and compared to sedentary controls. However, the increase was abolished by acute L-NAME given 30 minutes before blood flow measurement. This, together with pressure measurements, suggested that all of the training-induced increase was derived from improved NO-dependent flow-mediated dilation upstream of the collaterals and reduced microvascular resistance below them [94]. Despite these negative findings, it is noteworthy that metabolic arterial dilation during increased tissue activity favors increased pulsatile and mean pressure in collaterals. If prolonged, this could increase oscillatory shear and wall stress, ie, could "exercise" collateral endothelial and mural cells. This may induce collateral remodeling in healthy tissue. Zbinden *et al.* reported that a marathon runner free of angiographic CAD evidenced an increase in CFI_p after 6 months of aerobic training (although lower arterial pressure may also have factored into this) [95]. None of the above studies measured collateral diameter after exercise training—a metric that could be obtained in experimental animals.

In line with the above concept, coronary perfusion occurs predominantly during diastole, when coronary resistance vessels are partially dilated due to reduced extravascular compression and smooth muscle tone. Thus, prolonging diastole could enhance hemodynamic stimulation of collateral endothelial and mural cells, and like the possible effect of exercise, promote collateral outward remodeling. Patel *et al.* [96] retrospectively studied angiographic collateral scores in acute MI patients with heart rates ≤ 50/min (30 patients) vs. ≥ 60/min (31 patients). They found a significantly greater proportion with sinus bradycardia had well-developed collaterals than the control group. Whether this could also occur to native collateral diameter in healthy individuals is not known. In animal models, bradycardia induced by either pacing or alinidine treatment, increased capillary density in hearts, with potential involvement of elevated levels of angiogenic factors such as VEGF, bFGF, etc [97-99]. Also, combining acute coronary artery ligation or ameroid-mediated progressive occlusion with drug-induced bradycardia in rat, Dedkov, Tomanek *et al.* [100] observed increased arteriolar length density, improved regional coronary perfusion, and better preservation of cardiac function. The authors postulated that increased myocardial preload (stretch) and/or enhanced perfusion augments the native collateral circulation [100-102], possibly by increasing collateral remodeling. Likewise, aortic balloon pump and external counterpulsation (ECP), which favor increased coronary perfusion during diastole, improve collateral-dependent myocardial perfusion (see Chapter 12). It would be interesting to know if these intriguing findings could be extrapolated to native collaterals, ie, if bradycardia or ECP (or exercise) could augment collateral diameter in healthy individuals at risk from poor native abundance.

Genetic and environmental contributions to variation in arteriogenesis

Extensive research examining "environmental" factors, such as disease processes and risk factors (diabetes, smoking, etc), physiological mechanisms (eg, leukocyte homing, cytokines, growth factors) and therapeutic approaches (eg, protein, vector-delivered, cell-based), for effects on collateral remodeling has been reviewed recently [24,25,52-57] (see also other Chapters in this book) and will not be discussed here. Instead, our comments below address recent evidence that genetic factors can also impact arteriogenesis (see also Chapter 7 regarding epigenetic contributions). Scholz and co-workers first called attention to this possibility in their seminal study showing that recovery of perfusion after femoral artery ligation differed significantly between C57BL/6 and BALB/c mice [1]. This was subsequently confirmed [14,17,66,103-106], including studies that differentiated collateral remodeling from differences in angiogenesis, arterial pressure and native collateral extent—factors that affect recovery of perfusion [14,17]. The influence of genetic background on arteriogenesis has recently been further defined in the 15-strain mouse study of Zhang *et al.* [4]. Interestingly, findings in this and previous studies [14-16] examining both pial and hindlimb collaterals, indicate that genetic differences in collateral remodeling may be shared among the collateral circulations of different tissues, suggesting similar mechanisms direct collateral remodeling in different tissues. However, this may not be the case for flow-mediated outward remodeling of collaterals versus arteries; the order of mouse strain responsiveness reported in two studies of carotid artery remodeling do not agree with our findings for collateral remodeling: The amount of flow-mediated outward remodeling of the carotid was A > AKR > FVB/N > SWR > C57BL/6 > C3H/He > SJL > DBA/2 > BALB/c > 129/Sv in one study [108; measured 4 weeks after complete left carotid ligation] and was C57BL/6 > FVB/N > DBA/2 > SJL = C3H/He in another study [109; 2 weeks after partial left carotid ligation). In contrast, outward remodeling of pial collaterals after MCA occlusion was: SWR > A > AKR > C3H/He > CBA2 > KK/HI > 129S1/Svlm > NZW/Lac > NOD/ShiLt > BALB/cBy > C57BL/6 = SJL = DBA/2 > C57BLKS = FVB/N [4]. In addition, collaterals grow vigorously in the longitudinal direction during remodeling, compared to conduit artery remodeling (however, this increase in collateral tortuosity increases resistance). Together, the above findings demonstrate that genetic polymorphisms among individuals not only affect baseline extent of the native collateral circulation available for remodeling, but also affect activity of the arteriogenic pathways and/or responsiveness of collaterals to endogenous and exogenous arteriogenic molecules and, potentially, therapies.

Concluding comments – More questions than answers

The importance of the native collateral circulation is beginning to come into focus. Several unanswered questions, in addition to those stated above, warrant mention. Does the native collateral circulation serve a function in healthy tissues? Does it provide an adaptive advantage when abundant? Arterioles commonly branch off of collaterals in brain (eg, Fig. 2) and other tissues. In fact, pial collaterals in 15 strains of mice, including strains with only one or two collaterals, give rise to on average ~3 penetrating arterioles per collateral, regardless of strain [4]. So collaterals can serve as arteriole "scaffolds" across the collateral zone, supplying the surrounding tissue. It is possible that collaterals in tissues, particularly those subjected to mechanical activity (eg, muscle, intestine), insure better overall oxygenation under all functional states. For example, cerebral pial collaterals may contribute importantly to the well known and important "steal" behavior so prominent in this circulation. However, unlike the general and lymphatic circulations, the collateral circulation is clearly not required. For example, the distance between the crowns of the MCA and ACA trees in SWR and BALB/c strains—strains with almost no pial collaterals—are comparable to other strains with abundant collaterals. The general circulation of these strains apparently solves the dearth of collateral scaffolding by forming more extensive intracerebral terminal arteriolar trees to perfuse the cerebral parenchyma that underlies the would-be pial collateral zones. But does "fitness" then follow collateral extent? Unfortunately, it is not clear from existing phenome databases whether the mouse strains that we have identified with poor collateral extent perform more poorly in exercise or mental (or digestive?) tests.

Why is there such wide genetic variation in the collateral circulation, compared to the general and lymphatic circulations? This may arise because the collateral circulation is not *required* for survival. It thus may not have evolved redundancies that compensate for small (or large) changes in expression or protein function of collaterogenic genes—whether occurring by naturally arising gene variants or by epigenetic or environmental mechanisms—that have evolved in pathways governing formation of the general and lymphatic circulations. For the same reason, little selective pressure may exist to remove deleterious mutations in collaterogenic genes within a population. Of course, the collateral circulation does aid survival and fitness when obstructive disease strikes. However, these diseases and adverse environmental effects commonly arise or accumulate over time, well after the peak reproductive years.

At least in mice, the primary collateral plexus forms in a narrow and relatively late embryonic window. And these nascent collaterals then mature in a similarly narrow postnatal window. Are the signaling pathways governing these processes particularly susceptible to environmental disturbances? Are collateral endothelial cells specialized to insure that these vessels persist despite the adverse local hemodynamic environment? Are they specialized to direct the robust radial and longitudinal collateral remodeling in occlusive disease? Answers to these questions await us.

A better understanding of how collaterals form and are maintained—and the genetic, epigenetic and environmental factors that affect these processes—may lead to new approaches to recognize, prevent and possibly treat collateral insufficiency before or after occlusive disease strikes. Moreover, since native collateral number and diameter vary widely in mice and apparently also in humans, the failure to stratify patients in previous clinical trials of angiogenic/neovascular treatments may have contributed to the poor outcomes. Stratification of test groups according to pre-existing collateral extent may aid future trials. As well, patients with scarce native collaterals might benefit less from therapies aimed at augmenting collateral remodeling. Finally, the development of less invasive assessments of collateral extend could allow assignment of risk severity for stroke, CAD and PAD in healthy individuals, and aid adoption of preventive lifestyles or other measures.

In conclusion, the collateral circulation—at least in the cerebral circulation of the mouse, forms initially as a plexus late in embryogenesis, after the general arterial-venous circulation has formed. This plexus then undergoes pruning and maturation early postnatally. Genetic background has dramatic effects on these processes. Individual differences in the extent of this circulation may arise from natural polymorphisms that alter

expression of genes driving collaterogenesis, as well as environmental factors impacting formation and maintenance of these unique vessels. A more thorough understanding of collateral biology is not only fundamental, but may also help identify genetic variants and signaling pathways underlying differences in collateral abundance in humans. Such information may help in the development of therapies to promote formation of new collaterals in individuals that have or are at risk for occlusive disease.

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Chapter 2. Formation of Collateral Arterial Networks: Insights from the Developing Embryo

Ferdinand le Noble

Abstract: Arteriogenesis, the outward remodeling of preexisting small collateral arterial networks, occurs as a response to vascular occlusion or stenosis and importantly determines the clinical outcome of ischemic cardiovascular diseases. Distinct differences exist between animal strains, and patients with regard to collateral development. Besides differences in the efficiency of collateral recruitment and maintenance, this may also be explained from differences in the arterial branching architecture and number of pre-existing collaterals, prior to the occurrence of arterial occlusion. Since the branching architecture of the major arteries and their side-branches is established before birth, and during early post-natal stages, we postulate that conditions during early life, contribute to establishing the native collateral arterial network. In higher vertebrates, formation of branched vascular networks during embryogenesis involves a genetically hardwired component regulated by neural guidance genes and members of the notch signaling family, as well as a hemodynamic component. For establishing a stable branching architecture, the interaction of hemodynamics and neural guidance genes is of central importance. Hemodynamic forces can control the global branching architecture of the arterial network, vessel dimensions, and maintenance of vessel identity by regulating neural guidance genes. Neural guidance genes control arterial-venous identity and mediate guidance events that control and fine-tune positioning of growing vessels. Here we want to address the molecular pathways controlling arteriolar branching architecture during embryogenesis, in the context of the formation of preexisting collateral networks, and highlight the conserved function of molecules relevant for arterial differentiation in the embryo, during adult arteriogenesis.

Introduction

Arteriogenesis, the formation of a collateral artery network to bypass a stenosis, is physiologically the most relevant process for restoring perfusion to hypoperfused or ischemic vascular beds. Although the functional significance of arteriogenesis is appreciated, its biology is still poorly understood. The recent failures of clinical therapeutic angiogenesis trials aimed at stimulating vascular growth in ischemic cardiovascular diseases emphasizes the need for a more rational approach to this area of clinical cardiovascular medicine. Both clinical experience, and experimental analyses in pigs, dogs, rats and mice uncovered distinct differences between species, animal strains and between patients with regard to spontaneous collateral development, suggesting involvement of a strong genetic component. The nature of the genetic differences in arteriogenesis responses may involve variability in responsiveness to hemodynamics (flow/pressure), the ability to recruit blood derived cells (macrophages/monocytes/progenitor cells), or anatomical variability in arterial branching and number of preexisting collateral arteries. Clinical and experimental evidence indicate that hemodynamic forces exerted by blood flow, are crucial to initiate and propagate collateral growth. The molecular pathways linking the biophysical signals exerted by arterial flow with arterial growth are only partly characterized but involve activation of molecules relevant for controlling vascular tone (including nitric oxide, Klf2), vessel wall remodeling (matrix metalloproteinases), members of the Wnt-signaling pathway, VE-Cadherin, VEGF receptors, and the Notch signaling pathway (see the chapter by Limbourg). Clinical interventions aimed at stimulating hemodynamic forces in patients suffering from arterial stenosis show the first positive results and are discussed in more detail in Chapters 12 (Buschmann). In addition, experimental evidence suggests that growing collaterals are in an inflammatory state. The remodeling process associates with increased expression of adhesion molecules (ICAM-1, VCAM-1), chemoattractants (including MCP-1), supporting attraction, and invasion of circulating cells. Especially circulating monocytes/macrophages appear to positively affect arteriogenesis. Therapeutic interventions with GM-CSF have shown that stimulating influx of monocytes associates with increased arteriogenesis, but unfortunately also adversely affects atherosclerotic plaque progression, a major risk factor for developing arterial occlusion. Also application of various progenitor cell types have been shown to give a functional flow improvement to hypoperfused areas albeit at the risk for developing tissue neoplasia or

tumors. Therapeutic interventions with such agents are discussed in Chapters 11 (van Royen). Besides hemodynamics and inflammatory pathways, a third component relevant for arteriogenesis that has thus far not received a lot of attention is the arterial architecture and design of preexisting arterial collateral networks. In general, more preexisting arterial collaterals, with a larger vessel caliber, predicts a more efficient recruitment and enhanced flow delivery upon arterial stenosis. From the standpoint of prevention of ischemic damage, understanding how number and size of preexisting collaterals can be modulated is of clinical relevance. Especially in those organs where arterial occlusion results in severe end-organ damage, for example in the brain, and where spontaneous collateral development or pharmacological stimulation maybe to slow, optimizing collateral circulations prior to stenosis, is an intriguing therapeutic challenge. Recent experimental evidence provided by the group of Faber (see contribution in Chapter 1) has clearly shown a direct association between cerebral arterial branching architecture and stroke volume upon occlusion of the mid-cerebral artery. The challenge is to find genetic loci that link vascular architecture with improved collateral flow and clinical outcome after arterial stenosis. Since the architecture of the major arteries and their side branches is established at time of birth, some genetic components relevant for collateral arteriogenesis, maybe operational during fetal life. From developmental biology point of view, the outstanding questions are therefore: a) when are preexisting collateral arterial networks formed, b) which molecular pathways are involved in determining the number, size and maintenance of preexisting collateral arteries, and c) how does their functionality arise (type and amount of smooth muscle cells, wall dimensions, capacity to dilate/constrict). Extensive research during the last decade has led to the discovery of several novel cellular processes and molecular pathways, governing growth of arterial-venous networks during embryogenesis. Here, these new developments will we discussed in the context of the collateral arteriogenesis, and potential therapeutic strategies.

Choice of arterial patterns relevant for collateral arteriogenesis

The potential contribution of the arterial branching pattern in arteriogenesis can be demonstrated based on two examples (Fig. **1**).

In one case (Fig. **1**, left panel) we consider a completely dichotomous arterial branching pattern, as for example can be observed in the coronary system. The other case (Fig. **1**, right panel) is an arterial pattern with many arterial-arterial connections, as for example can be observed in the intestinal circulation. In the arterial tree with a dichotomous branching pattern, exposed to arterial occlusion, the lack of preexisting arteriolar-arteriolar connections, prevents the fast recruitment of collateral pathways, leading to insufficient blood flow delivery to sites distal of the occlusion in the acute phase after stenosis. Pending the oxygen needs of the target organ, this scenario can lead to severe tissue damage and loss of organ function. Theoretically, it may be argued that due to the occlusion and redistribution of blood flow, adaptive remodeling occurring at the capillary level induces formation of collateral connections. Some of the most distal small arterioles are interconnected via the capillary network, and increasing the caliber of such connections may facilitate collateral flow. Since arterial stenosis results in shunting of flow to other parts of the arterial tree, and increased blood flow is a stimulus for capillaries to be “upgraded” into small arterioles, such process may indeed result in the formation of small collateral connections in distal areas. However, this adaptive remodeling process occurs relatively slow, and it is therefore debatable whether it has physiological relevance during the early phase after arterial occlusion. In contrast, organs with an arterial system with many preexisting arterial-arterial connections (Fig. **1**, right panel), most likely don’t suffer form loss of organ function after occlusion. In this case, after occlusion, many preexisting pathways prevent complete loss of flow to the areas distal of the occlusion. Of course, the efficacy of flow delivery in this scenario depends on the caliber of such arteriolar connections and their contractile status. Thus far, most experimental evidence indicates that the redistribution of flow after arterial occlusion, results in increased shear stress in collateral arterioles concomitantly with activation of vasodilatory pathways (see chapters on NO by Schaper, Unthank), collectively resulting in a positive outward remodeling of the preexisting arteriolar connections. Pending on the site of occlusion, pressure gradients may also change, and modulate smooth muscle cell responsiveness to growth factors, or recruitment of progenitors into the growing collaterals. The differential contribution of shear

and wall stress are discussed in more detail elsewhere in this book (see chapter by Schaper). Since in general the number of side-branches in the proximal part of the arterial tree is limited, the principle of recruitment of preexisting collateral anastomoses functions more efficiently in parts with numerous side branches, hence the distal parts of the arterial tree. For occlusions occurring in the most proximal part of the arterial, other interventions for restoring blood flow should be considered.

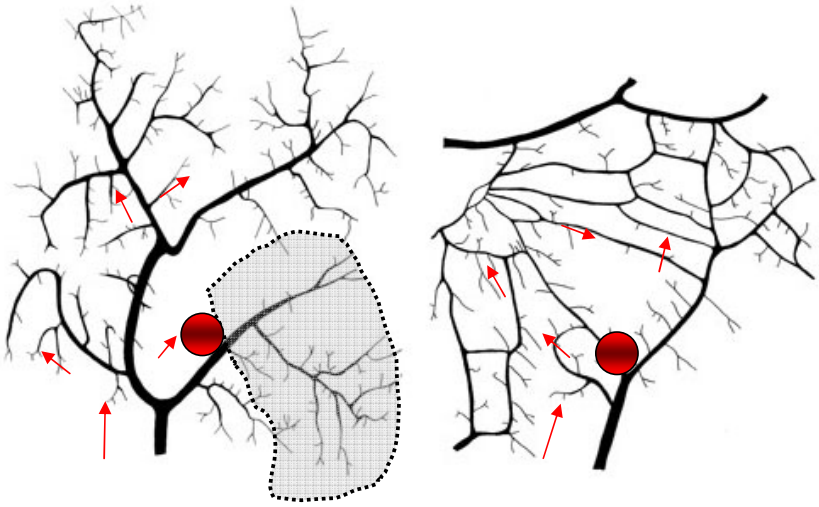

Figure 1: Effects of branching architecture on blood flow distribution after arterial stenosis. Left panel: dichotomous arterial branching pattern without arterial anastomosis connections. Arterial stenosis indicated with red circle. The arterial blood flow direction after stenosis is indicated with red arrows. In this scenario, upon arterial stenosis, the absence of arterial bypass connections impairs flow delivery distal to the occlusion, resulting in a large area at risk (dotted line) for hypoperfusion and tissue ischemia. Right panel: arterial pattern with many artery-to-artery anastomosis connections. Arterial stenosis indicated with red circle. The arterial blood flow direction after stenosis is indicated with red arrows. In this scenario, blood flow can efficiently be re-routed around the stenosis. Pending on the speed of flow induced outward remodeling, and the functionality of the preexisting arterial-arterial connections, this scenario is likely to result in a small area at risk.

Ontogenesis of arterial collaterals

Collateral arteriogenesis is defined as the positive outward remodeling of preexisting collateral arterial connections that bypass a stenosis resulting in restoration of blood flow to the hypoperfused region. In addition, in response to arterial occlusion, de novo formation of blood vessels involving angiogenesis, and “upgrading” of capillaries into small arterioles may occur. Recruitment of preexisting arteries/arterioles implies that they have to be present at time of occlusion. Heterogeneity exists between organs with respect to collateral arterial network development, but the organ specific molecular cues involved herein remain to be uncovered. Recent experimental evidence obtained from adult mouse brain, has clearly shown that the positive association between number of preexisting arteriolar connections in the cerebral circulation, and stroke volume upon arterial occlusion, involves a genetic component. Using artery specific reporter mice, we could show that such arterial collateral connections are already detectable during neonatal stages, implying that they were established before birth (Fig. **2**; LeNoble, unpublished). We therefore focus on the genetic regulation of arterial patterning in the embryo.

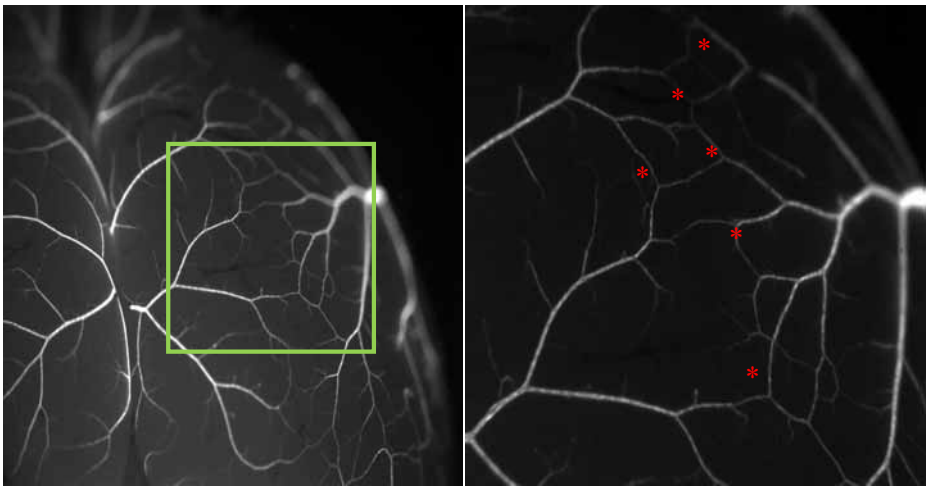

Figure 2: Arterial collaterals in the cerebral circulation of neonatal mice. Left panel: overview of the arterial branching pattern of the cerebral circulation in mice selectively expressing GFP in arteries. Note the connections between the ACA and MCA. Right panel: detail of boxed area in the left panel. Note the numerous preexisting arteriolar-arteriolar connections (asterisk) in the distal part. In case of an occlusion occurring in the MCA, blood flow from the MCA can be shunted through these collateral anastomoses, and potentially limit perfusion deficits. (LeNoble, unpublished)

Vascular development during embryogenesis

The formation of a properly branched vascular system is critical for embryo development and survival. Two successive processes called vasculogenesis and angiogenesis, achieve blood vessel formation during embryonic development. Vasculogenesis is the in situ differentiation of angioblasts and endothelial cells from mesenchyme [1,2], and their coalescence into tubes of the primary vascular plexus. This process is responsible for the formation of the central axial vessels (dorsal aortae, and the cardinal veins), and a meshwork of capillaries that collectively can accommodate the cardiac output, once heartbeat commences.

Expansion of the primitive vascular system formed by vasculogenesis occurs through sprouting and intussusception angiogenesis [3]. These distinct angiogenesis processes lead to the remodelling of the primary vascular plexus into a hierarchical vascular tree, consisting of branched arteries, capillaries, and branched veins [4]. Sprouting angiogenesis involves the budding, and extension of new segments from preexisting vessels, that grow into previously avascular areas. The main driving forces for this process are gradients of vascular endothelial growth factor (VEGF) and hypoxia. Sprouting angiogenesis will be discussed in more detail in the next paragraphs.

Intussusceptive angiogenesis is a relatively recently discovered mode of blood vessel formation, and occurs by internal division of the preexisting capillary plexus without sprouting. In the first step of intussusceptive angiogenesis, the capillary plexus expands by insertion of numerous transcapillary tissue pillars, so called intussusceptive microvascular growth (IMG). One important difference with sprouting angiogenesis is that the IMG process is fast, and can give rise to new capillary segments within 4-8 hours, does not require substantial changes in endothelial proliferation, and is less dependent on vascular endothelial growth factor (VEGF), when compared to sprouting angiogenesis. IMG leads to a considerable increase in capillary surface area, and has been well described in the developing lung. In the second intussusception step, a vascular tree with distinct arterial and venous segments arises from the primitive capillary plexus as a result of intussusceptive pillar fusion, a process called intussusceptive arborisation (IAR). During IAR, rows of pillars are transformed to tissue septa by pillar reshaping and pillar fusions. This pillar reshaping

demarcates prospective supplying and collecting vessels, and leads to the lifting off and extraction, of the new vascular entity into an adjacent (feed/draining) vessel layer.

In addition to IMG and IAR, a third special form has been described, intussusceptive branching remodelling (IBR), which is involved in shaping branching angles of developing vascular networks. It is postulated that IBR results in optimization of branching angles, and lumen dimensions at vessel bifurcations contributing to minimizing power consumption and constant shear stress throughout the vascular system. IBR occurs rapidly (within hours) in response to changes in flow.

While the functional relevance of intussusceptive angiogenesis is recognized, still little is known about the detailed molecular and cellular events regulating this important process. Also the extent to which different organs use sprouting or intussusception for vascular network development remains to be established. Given the role of IAR in establishing arterial segments from capillary networks, and the potential regulatory role of blood flow herein, it may have importance for shaping pre-existing collateral networks.

Recruitment of pericytes and smooth muscle cells, around the endothelial cell layer, completes the anatomical formation of the vascular wall. During embryogenesis, vascular smooth muscle cells may originate from the ventrolateral mesoderm and mesectodermal derived neural crest cells. It is suggested that the vascular smooth muscle cells in the media of the large central arteries arise from neural crest cells. Subsequently, during fetal development, smooth muscle cells mature, acquire functional properties, and attract (sympathetic) nerves allowing central control of vascular tone. The ontogenesis of organ characteristic features of smooth muscle cell structure and function remains to be determined.

Regulation of vessel identity and branching morphogenesis

The branching hierarchy of the major vessels is highly stereotyped within and across species. Secondary sprouts branching from the major axial vessels like the intersomitic vessels, or the main arteries penetrating different organs and the limbs, form at anatomically designated sites. In general, the gross vascular anatomy of the mouse, chick or zebrafish embryos is characterized by highly reproducible branching patterns. In the more distal areas, embryonic vessel development is highly dynamic and subjected to active remodelling. Vessel segments can be disconnected and reconnected to facilitate growth of new vascular beds, or pruned pending the local hemodynamic conditions. Furthermore, environmental cues derived from the expanding organs contribute to patterning. Oxygenation of developing embryonic tissues is one of the determining factors for vessel patterning. Regions of hypoxia generate strong attractive signals for angiogenic sprouts, which are lacking in organs with normoxia or hyperoxia. In peripheral tissues, the patterning of arteries and nerves is often congruent. In the skin of the mouse embryo limbs, nerves can supply growth factors allowing local differentiation and patterning of small arterioles.

During the last decade, extensive research addressing the formation of vascular networks has shown that endothelial cells consists of genetically and morphologically distinct populations of cells essential for establishing arterial-venous identity, and branching. Depending on the stage of development and the vascular bed investigated imprinting of vessel identity and branching can be regulated by two distinct mechanisms: 1) genetic hardwiring of vessel positioning and identity, 2) flow controlled vascular patterning events and maintenance of vessel identity [5]. The expression of neural guidance genes in vessels plays a fundamental role in both mechanisms [6]. Neural guidance genes were originally described in *C. elegans* to mediate various aspect of neuronal development. In 1998, the group of David Anderson was the first to provide genetic evidence showing that neural guidance molecules of the ephrin family can specify arterial-venous identity in vessels, essential for remodelling of capillaries into arteries and veins during embryogenesis. They showed that reciprocal interactions between ephrinB2 selectively expressed in arteries, and EphB4 selectively expressed in veins, triggers separation of arterial and venous domains, allowing arterial-venous network development. These results implied, for the first time, that genetic programming of arterial and venous identity in endothelial cells is essential for establishing proper vascular network morphogenesis. In 2003, the group of Holger Gerhardt showed that angiogenic sprouts also consist of genetically distinct populations of cells. Angiogenic sprouts are spearheaded by so

called endothelial tip cells. Tip cells are migratory and extend numerous filopodia to sense the local microenvironment and guide the direction of sprout growth. Tip cells are followed by so called stalk cells. These stalk cells proliferate, form a vessel lumen, essential for local flow delivery. While VEGF is the main activator of tip cell differentiation and angiogenic sprouting, tip cell filopodia can also express neural guidance receptors, controlling tip cell behaviour and sprout positioning. The function of neural guidance molecules in the tip cells shows striking similarities with their role in the developing neural system. Growing axons are guided by axonal growth cones, which express guidance receptors that are capable of sensing attractive and repulsive cues from the surrounding extracellular compartment, controlling the direction of axonal growth. The functional equivalent in the vascular system is the endothelial tip cell (for more reviews see [5,6]).

Taking into account that vessels arose later during evolution than nerves, the concept emerged that the developing vascular system has coopted growth control mechanisms from the neuronal system to mediate vascular guidance events and specify arterial-venous cell fate in blood vessels. This concept is now firmly established, and functional roles for several ligands and receptors of the main neural guidance gene families, in vascular development, have been reported.

Endothelial tip and stalk cells:

Endothelial tip cell behaviour and function have been well assessed in the mouse embryo hindbrain, mouse neonatal retina, and in zebrafish embryo intersegmental vessel branching. The concepts presented here are mainly derived from observations in these model systems. Here we will address why some endothelial cells become tip cells, and how tip cell guidance is achieved. In the mouse retina, VEGF is the major player inducing tip cell differentiation, and tip cells express VEGF-receptor-2 (Kdr/Flk1) on their filopodia, essential for sensing extracellular VEGF [7]. Local tissue growth in the expanding retina is accompanied by hypoxia and local production of VEGF by astrocytes, resulting in a gradient of VEGF and the attraction of angiogenic sprouts towards the hypoxic regions. Scavenging local VEGF with soluble Flt1 (soluble VEGF-receptor-1), or injection of VEGF-receptor-2 neutralizing antibodies, induces tip cell filopodia retraction and loss of tip cell numbers [7]. The continuous presence of a VEGF gradient is necessary to maintain filopodia, and sprout extension towards the hypoxic region. When sprouts subsequently fuse and start to deliver flow and oxygen, hypoxia, hence the trigger for VEGF production, disappears, and sprouting stops. VEGF is spliced in several isoforms with distinct effects on sprouting extensions. The magnitude of attraction, short or long range, varies between the VEGF-isoforms and is related to the extracellular matrix binding capacity [8], and affinity for the VEGF co-receptor and guidance molecule neuropilin-1 [9]. The diffusible form of VEGF seems to act as a short range attractor, whereas the extracellular matrix bound VEGF isoforms act as long range attractors. Interestingly, ectopic VEGF perturbs extrinsic vessel pattering cues, suggesting that the VEGF gradient provides insufficient information for sprout guidance in the immediate vicinity of the parent vessels, and that additional cues, most likely involving soluble VEGF-receptor-1 (soluble Flt1), must exist. While membrane bound Flt1 is mainly expressed on stalk cells and assumed to repress tip cell identity in stalk cells, it was shown that developing mouse blood vessels produce sFlt1, coordinating local sprout guidance [10]. Soluble Flt1 is produced and secreted around the lateral base areas, immediately adjacent to the developing sprout. Here sFlt1 neutralizes VEGF, and sharpens the VEGF gradient towards the tip cell front region, hence providing a corridor for sprout extension pushing the sprout in the proper direction. Next to VEGF-R2, also VEGF-R3 (Flt4) is expressed strongly on tip cells and mediates angiogenic sprouting in mouse and zebrafish. In mouse, VEGF-R3 is highly expressed in angiogenic sprouts of retina and tumor vessels, and genetic ablation of VEGF-R3 signalling, or blocking of VEGF-R3 with neutralizing antibodies, reduces sprouting [11]. Moreover, stimulation of VEGF-R3 augments VEGF induced angiogenesis, even if VEGF-receptor-2 signalling is blocked.

While VEGF and VEGF receptors regulate tip cell behaviour, it has become clear that the Notch signalling pathway plays a central role in modulating tip – stalk cell differentiation. Recently, a series of studies addressing the molecular mechanism controlling specification of endothelial tip cells in mouse and zebrafish showed a key-role for Notch receptors

(presumably Notch-1) and the Notch ligands, Delta-like-4 (Dll4) and Jagged-1 (Jag1)[12-15]. Dll4 is predominantly expressed on endothelial tip cells, whereas the Notch receptors and Notch signalling mainly occurs on the stalk cells. Genetic deletion of one allele of Dll4 results in supranumerous amounts of endothelial tip cells, as evidenced by the increased expression of the tip cell markers Pdfgb, Flk1, Flt4, Unc5B, and apelin, augmented filopodia extensions, excessive angiogenic sprouting resulting in increased capillary network formation [12,13]. Pharmacological inhibition of Notch signalling using compounds that inhibit gamma-secretase (for example DAPT), hence, the release of notch intracellular cleaved domain (NICD, which is normally released after stimulation of Notch receptors) phenocopies the vascular phenotype observed in the genetic models. In zebrafish, knockdown of Notch1b and rbpja (orthologue of mouse Rbpj), and treatment with DAPT also results in excessive branching, confirming the potential role of Notch signalling in restricting tip cell differentiation, and angiogenic cell behaviour. Interestingly, the Notch ligand Jagged-1 (Jag1) appears to act as an antagonist of Dll4-Notch signalling in tip cell differentiation. Using loss and gain of function models in mice, the group of Ralf Adams has shown that endothelial specific loss of Jag1 leads to impaired retinal angiogenesis and fewer tip cells [16]. In contrast, overexpression of Jag1 in retinal endothelium resulted in increased angiogenesis and more tip cells. Expression analysis of endothelial cells isolated from the retina showed that Notch signalling is more active in the absence of Jag1, concomitantly with an inhibitory effect on vessel growth. At the cellular level, this response depended on the presence of Fringe, which function as modifiers of Notch receptors, controlling Dll4/Jag1 signalling efficiency. Expression of Jag1 in stalk cells prevents that co-expressed Dll4 can activate Notch in these endothelial cells. This activity of Jag1 depends on Fringe, which reduces Notch activation by Jag1, enhances signalling by Dll4, thus leading to a competition between an agonist (Dll4) and antagonist (Jag1). Since Jag1 is predominantly expressed on stalk cells, and Dll4 on tip cells, such complementary expression patterns support a model in which Jag1 negatively regulates Dll4 mediated Notch activation. In stalk cells, residual Dll4 expression that could potentially block angiogenesis is present, and by antagonizing these signals, Jag1 may promote stalk cell activation, allowing sprouting behaviour and VEGF responsiveness.

The physiological concept that arises from these studies is that endothelial cells expressing VEGF-receptor-2, in response to a VEGF gradient can start to express Dll4 on its cell membrane, and function as a tip cell. Dll4 subsequently activates (presumably) Notch-1 receptors on the adjacent stalk cells. The activation of Notch-1 suppresses tip cell differentiation in these stalk cells. As a result, only the cell that initially differentiated into a tip cell will remain tip cell, from the spear-head of the angiogenic sprout and grow towards the VEGF gradient, usually induced by local tissue hypoxia (associated with organ growth). This unique model thus results in limiting the amount of sprouts that respond to a hypoxic challenge. However, in this model, the default pathway for an endothelial cell is considered to be tip cell. Hence, in the context of arteriogenesis and formation of preexisting collateral pathways, modulation of any signalling element of the tip-stalk cell differentiation cascade, may influence the design of the natural occurring bypass circulation!

The principle in which a differentiating cell represses the differentiation of adjacent cells, as occurs during tip-stalk cell differentiation is called lateral inhibition. This growth principle is well established in the developing nervous system furthermore highlighting the similarities between both systems.

Tip cell guidance and neural guidance molecules

Neural guidance gene function has been well documented in the nematode. Genetic screens of nematode locomotor (uncoordinated, unc) mutants led to the discovery of several ligands and receptors relevant for commissural and motor axon guidance, including unc5 and the ligand unc-6, netrin. Studies in higher vertebrates subsequently showed that their function in the developing nervous system is preserved. Surprisingly, recent evidence shows that for many neural guidance genes, their function even extends to the growing vascular system. This led to the concept that during evolution, neural guidance gene function has been co-opted by the developing vascular system to mediate guidance events. To what extent neural guidance genes can play a functional role in shaping collateral bypass circulation has not been explored well. In the developing nervous system, axonal guidance depends on the growth cone, the

migrating distal tip of the growing axon. These growth cones send out numerous filopodia and lamellipodia that scan the local environment for attractive and repulsive guidance cues. In the growing vasculature, the functional equivalent of the axonal growth cone, the endothelial tip cell, also expresses neural guidance receptors determining sprout positioning. This has been best documented for UNC5B and its ligand netrin-1 [6,17].

In mice the UNC5B receptor is selectively expressed in arteries and endothelial tip cells. Genetic ablation of UNC5B resulted in increased capillary sprouting in the hindbrain, intersomitic vessels and retina. In the retina and hindbrain, administration of netrin-1, a ligand for UNC5B, resulted in filopodia retraction. Endothelial tip cells exposed to netrin-1 in vitro moved away from the netrin-1 gradient. Moreover, morpholino mediated knockdown of the zebrafish orthologues for UNC5B or netrin-1 resulted in misguidance of intersegmental vessels. Aberrant path finding in both UNC5B and netrin-1a morphants occurred at the level of the horizontal myoseptum (the natural occurring source for netrin-1), instead of extending dorsally. As a result, vessel branching was increased, resulting in a phenocopy of the vascular patterning defect observed in the UNC5B mouse mutant [17]. Interestingly, loss of function studies in mouse and zebrafish indicate that the plexinD1-semaphorin3E signalling pathway may act in a similar way albeit at different positions in the growing vascular tree [18,19]. It is therefore postulated that the developing embryo has several tissue specific guidance checkpoints that control local branching morphogenesis. At present the full repertoire of guidance genes is not known.

Regulation of arterial-venous identity in blood vessels

After the formation of the initial vascular plexus through vasculogenesis and angiogenesis, vessels have to differentiate into arteries and veins in order to allow proper perfusion [20]. Arteries and veins in adult networks differ considerably in morphology and function. These differences arise during embryogenesis and involve neural guidance genes expressed in the vascular system.

Several signalling molecules have been discovered, which differentially label arterial or venous endothelial cells from early developmental stages onward, prior to the onset of circulation. Many of these molecules are also expressed in the nervous system, where they regulate cell fate decisions and guide migration of neuronal precursors as well as of developing axons [21-24]. Arterial EC in chick, mouse and zebrafish selectively express ephrin-B2, neuropilin-1 (NRP-1) and members of the notch pathway, including notch3, DLL4, and gridlock [25-31]. Other molecules are specifically expressed in the venous system, most notably EphB4, the receptor for arterial ephrin-B2 [32], neuropilin-2 (NRP-2) [25,33] and Coup-TFII [34]. Mutations of the ephrinB2 tyrosine kinase and its receptor EphB4 both lead to early embryonic lethality around E9.5 [30,32,35,36]. Remodelling of the primary vascular plexus into arteries and veins was arrested in both receptor and ligand mutants. These findings suggest important roles for ephrinB2/EphB4 interactions on arterial and venous endothelial cells, respectively.

The currently proposed genetic mechanism regulating arterial identity involves VEGF, binding to its heterodimeric receptor VEGFR-2 and neuropilin-1, activating the Notch signalling pathway, Hey transcription factors, leading to induction of ephrinB2 and suppression of EphB4 [12,37]. During normal development, expression of COUP-TFII in venous endothelium suppresses neuropilin-1 and inhibits Notch signalling and the expression of artery specific genes [34]. Without neuropilin-1 and Notch signalling, venous markers like EphB4 are expressed and venous identity maintained. Ablation of COUP-TFII in endothelial cells enables veins to acquire arterial characteristics including expression of ephrinB2, neuropilin-1 and Notch signalling molecules. However, it was noted that the acquisition of the arterial identity in veins of COUP-TFII mutant mice was not sufficient to convert mutant veins fully into arteries suggesting additional control mechanisms including cells in the vessel wall [34].

Chick quail chimera studies [27] and in vitro endothelial-smooth muscle spheroid cell culture experiments [38] indeed indicate a potential role for vascular smooth muscle cells in molecular instruction of endothelial cells with an arterial identity. Alternatively, such cues can also come from the perivascular nerves as has been demonstrated for sensory nerves in the

developing mouse skin vasculature [39]. However not all arteries become innervated by autonomic nerves, and most arteries differentiate before development of nervous innervation.

Taken together these data indicate that, before the onset of heart beat, genetic hardwiring of arterial-venous differentiation is essential to allow proper vascular development. Genetic hardwiring of arterial-venous identity is essential for the proper development of the in- and outflow tract of the heart, and the establishment of the aorta and cardinal vein which will allow delivery of cardiac output to the developing organs once the heart starts beating.

After the start of heartbeat and generation of cardiac output, the developing vascular system shows a high degree of endothelial cell plasticity. Using a combination of in vivo molecular imaging and vascular grafting studies we showed that endothelial cells are not genetically committed to their initial phenotype but plastic and adapt their identity continuously depending on local epigenetic cues including hemodynamics [40]. In addition we obtained evidence showing that the global patterning of arteries and veins in peripheral organs like the yolk sac depends on hemodynamics and local tissue compliance. We observed several novel morphological events essential for arterial-venous differentiation and branching during embryogenesis. These included 1) flow driven fusion of small calibre vessels into large tubes, a process accounting for the formation of the large vitelline arteries and veins, 2) selective disconnection of endothelial cells from the arterial system and reuse of these endothelial cells to fashion growth of the venous system, 3) shear stress driven guidance of lumenized vessel sprouts. We could show that in the absence of cardiac output and active flow perfusion of the yolk sac, despite the expression of arterial markers like ephrinB2, AV differentiation and patterning would not take place. In addition, perfusing an embryonic artery with venous blood, transformed this artery genetically and morphologically into a vein. Arterial markers like neuropilin-1 and ephrinB2 were downregulated whereas venous markers including neuropilin-2 were upregulated [40]. From these observations we concluded that hemodynamics control arterial-venous differentiation and are essential for the maintenance of AV vessel identity in the yolk sac vasculature.

Arterial – venous vessel plasticity is also observed in the developing trunk vasculature of the zebrafish embryo [41]. In early stage embryos, primary vessel sprouts grow between the somites to form the intersomitic vessel. This vessel subsequently branches laterally into the caudal and cranial regions to from the dorsal lateral anastomising vessel (DLAV). At this stage, the intersomitic vessel is connected to the aorta and is considered arterial, carrying arterial flow. During subsequent stage so called secondary sprouts that originate from the cardinal vein form. These venous sprouts grow parallel and in close proximity to the intersomitic arterial vessel. Occasionally, these venous sprouts fuse with the intersomitic arterial segment, which progressively loses its connection with the aorta as the intersegmental vessel turns into a vein [41]. As a consequence of the vein fusing with the arterial segment process, the flow direction in the intersomitic arterial segment is reversed. The exact cellular and molecular mechanism accounting for this process is not known. It is suggested that the fusion of veins with arteries is the result of a stochastic process and essential to balance local perfusion. Similar to the morphological events observed in the developing chick embryo, plasticity of arterial segments seems to be important to obtain functional perfused networks. To what extent such plasticity events occur in other organs during development remains to be elucidated.

Recent evidence shows that blood flow is also essential for driving vascular remodelling in the mouse embryo yolk-sac [42]. Using Mlc2a-/- mutant mice, which display a selective cardiac contractility defect impeding proper control of cardiac output, it was clearly demonstrated that without adequate flow, yolk sac remodelling does not occur. This could be attributed to the mechanical properties of the early blood, not the presence of circulation angiogenic factors or changes in oxygen carrying capacity [42]. Using elaborate erythroblast trapping techniques and injection of high molecular weight synthetic sugars, it was demonstrated that the mechanical force, normally imparted by the flow of circulating erythrocytes, is necessary and sufficient to induce vessel remodelling in the yolk-sac of early mouse embryo [42]. It remains to be tested whether erythrocyte mediated changes in viscosity also affect vascular remodelling in other model systems and pathological conditions.

The zebrafish embryo can generate the aorta, cardinal vein and segmental sprouts in the absence of perfusion. However, in some mutant fish, blood flow driven arteriogenesis can be

observed [43]. The *gridlock* mutant zebrafish embryo is characterised by permanent occlusion of the proximal aorta [44] caused by a mutation in the Hey2 transcription factor that is involved in arterial specification. In early development, as a result of this occlusion, the distal aorta does not carry flow. With time, gridlock mutant embryos recover blood flow in the distal aorta via communications with the intestinal vasculature [43]. This arteriogenesis response can be phenocopied by laser-induced proximal aortic occlusion. Pharmacological inhibition of nitric oxide synthesis using L-NAME attenuated collateral formation in *gridlock* mutants indicating involvement of nitric oxide. Morpholine knockdown of pu.1 to prevent myeloid development also reduced restoration of distal aorta flow [43], suggesting that analogue to the adult situation, the presence of myeloid cells may accelerate arteriogenesis [45]. Analysis of hypoxia markers including HiF-1α, revealed that in *gridlock* mutants collateral flow develops independently of tissue hypoxia or ischemia. Thus, collateral aortic blood flow in gridlock mutants was dependent on both nitric oxide and myeloid cells. More recent evidence shows that blood flow is also relevant for arterial growth in the cerebral circulation of zebrafish embryos.

Taken together increased levels of shear stress, as well as the independence from ischemia are three unique features in arteriogenesis, which are present in both, mammals as well as zebrafish, during embryogenesis and in the adult. These observations suggest that flow driven arteriogenesis is highly conserved.

Flow driven arteriogenesis in the chicken embryo

In the chicken yolk sac circulation, hemodynamic factors are important for the regulation of the expression of arterial-venous identity genes. In addition, we noted that changes in the arterial blood flow distribution evoked dramatic changes in the overall patterning of the vitelline arteries and veins (Fig. **3**). To understand the nature of this flow controlled arterial patterning, we performed detailed time-lapse imaging, and hemodynamic characterizations *in vivo*, and observed a striking resemblance with "adult arteriogenesis" including flow driven enlargement of small arterioles, and the use of pre-existing vascular segments to build the collateral arterial network [46].

We observed that the direction of outgrowth of the arterial tree, closely associated with the direction of the arterial blood flow distribution (Fig. **3B**) obtained after arterial occlusion. In normal embryos, cardiac output is distributed toward the left (LVA) and right (RVA) vitelline arteries (Fig. **3A**). In the posterior pole, the arterial blood flows into the vitelline vein plexus in this area (Fig. **3A, C**). Selective ligation of the RVA using a metal clip changes the blood flow distribution in the yolk-sac (Fig. **3B**). In the ligation model, the cardiac output is now distributed to the LVA, resulting in increased shear stress values throughout the LVA arterial tree. In response to exposure to elevated shear stress levels, LVA arterioles grow, resulting in a structural increase of lumen diameter. In the posterior pole of ligation embryos, flow is distributed towards the vitelline vein, and the terminal arterioles of the RVA. In the latter, the blood flow direction is reversed (Fig. **3B**, blue arrows) compared to control unligated embryos. The reason for this change in blood flow direction is that occluding the RVA, results in "low pressure" immediately distal of the occlusion. This will subsequently attract blood flow towards this area (it functions as a sink). This blood flow is attracted from the posterior pole, and the sinus vein, involving retrograde perfusion of pre-existing RVA segments. In the posterior pole, there is competition between blood flowing back to heart through the vitelline vein, and blood flowing back to the heart, via the pre-existing RVA arterioles.

With time, the amount of blood flowing to the RVA arterioles increases, whereas the amount of blood flowing back through the posterior vein (which is oriented perpendicular to the preferential flow direction after ligation) decreases. As a result of the reduction in blood flow, the posterior vitelline vein adapts with structural inward remodeling and is finally pruned. In contrast, the pre-existing vessel segments that are in line with the new flow direction, obtain more flow, show structural outward remodeling and extend toward the right hand side of the embryo. Thus, after ligation, the creation of a "low pressure point" at the occlusion site, now results in steal of blood flow from the posterior vein, resulting in a change in flow direction, ultimately evolving into flow mediated diameter and arterial patterning changes.

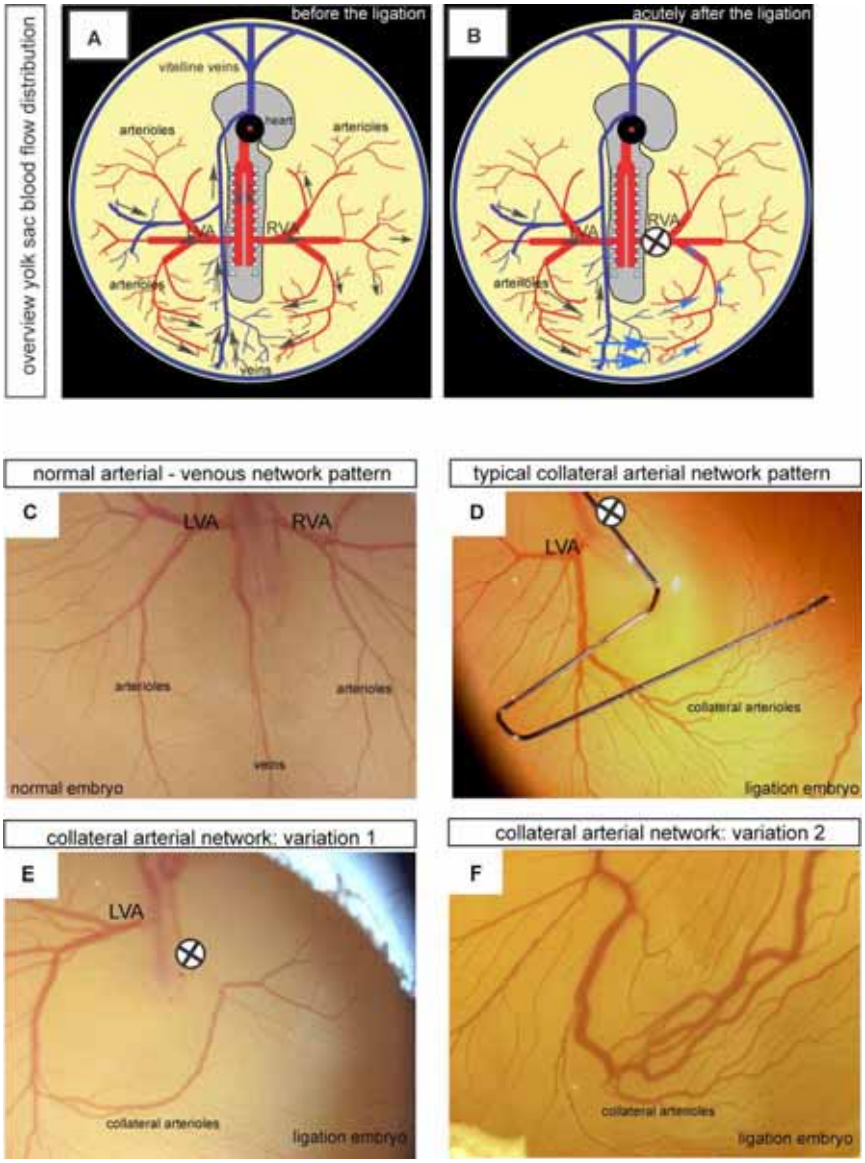

Figure 3: Flow distribution and formation of arterial collateral networks upon right vitelline artery occlusion in the chicken embryo. (A) Normal blood flow distribution. Note that the flow (black arrows) from the left (LVA) and right (RVA) vitelline artery, both drain into the vein located in the posterior area. (B) Blood flow distribution after selective ligation (circle) of the RVA with metal clip. Note that the blood flow from the LVA is distributed towards the vein, and the pre-existing arterioles of the RVA. The flow direction in the LVA arterioles is reversed (blue arrows). (C) Normal arterial-venous pattern in control embryos. (D) Typical collateral arterial network as observed 24 hours post-ligation of the RVA. Note that the arterioles branching from the LVA, followed the flow pattern (blue arrows in B), and extend across the previously venous domain in the posterior part, projecting towards the right hand side. The vitelline vein in posterior area, regressed. (E) Collateral arterial network pattern variation-1. (F) Collateral arterial network pattern variation-2, huge collateral arterioles developed in the posterior pole. (adapted from Buschmann&leNoble, [46].

These observations suggest, that in this organ, during this stage of embryonic development, the directionality in the arterial blood flow component may guide the global patterning of the arterial network at the macro level, as opposed to the tip cell mediated guidance events at the capillary, micro level. In our setting, exposureing the complete embryo to hypoxia did not accelerate arteriogenesis. In this setting (global hypoxaemia) arteriogenesis proceeded slower and associated with reduced cardiac function and tissue perfusion. This indicates that for the yolk sac vasculature, blood flow, and not oxygen availability per se, maybe the main driving force for vessel development, as opposed to endothelial tip cell sprouting which clearly involves a hypoxia driven (and VEGF gradient) component.

Another characteristic feature of "adult arteriogenesis" is the variability in the collateral arterial architecture among patient populations or between mouse strains. Similar observations were made in the chicken embryo ligation model. While in the majority of cases investigated, the collateral arterial architecture resembles the pattern presented in (Fig. **3D**), in some case (less than 1%) we noted alternative patterns as presented in (Fig. **3E**) (variation-1) and (Fig. **3F**) (variation-2). In the case of collateral architecture variation-1, collateral arterioles developed fast, and reconnected to the stem of the RVA, just distal from the occlusion. The collateral arterioles carried flow, and perfused the RVA in the normal forward direction (Fig. **3A**). The number of collateral arterioles appears smaller when compared to the typical collateral pattern (Fig **3B**). In collateral architecture variation-2, we noted the formation of comparatively large arteries. The pattern resembled, to some extent the collateral architecture obtained in the rabbit shunt model as developed by Eitenmueller and Schaper. Indeed, in our setting these large caliber collaterals were also high flow vessels. The mechanism accounting for this natural variability is not known but may involve a genetic component or specific aspects of the yolk sac vasculature at the time of ligation, which remain to be determined.

Collateral Arteriogenesis: from embryo to adult

The outstanding question in the field is how the recent advancements in understanding the molecular and cellular regulation of vascular development translate into the formation of functional collateral arterial networks that can be recruited after stenosis of a main feed artery. The research focus should therefore be at elucidating how the "embryonic" growth mechanisms can lead to patent collaterals.

Unfortunately, most studies addressing the "embryonic principles" in adult vascular development thus far focused on involvement in angiogenesis in ischemic tissue, or tumor growth. Since angiogenesis and (collateral) arteriogenesis differ considerably, alternative approaches aimed at quantifying the fetal contribution to branching patterning, maintenance of collateral circulations, and assessment of functional adaptive responses in such networks, including maturation of the vessel wall function should be considered. It is clear that an important part of vessel identity and arterial branching is regulated by genetically hardwired components. It is to be expected that any alteration in VEGF gradients, VEGF receptor expression and signaling, neural guidance molecules (UNC5B, PlexinD1), perivascular nervous innervation, as well as modifications in Notch receptors and ligands may affect arterial specification, and branching morphogenesis. Indeed, recent studies by the group of Faber has shown that VEGF and Flt1 are determinants of native collateral formation [47,48].

Another unsolved issue is the maintenance of potential collateral arteriolar side-branches that branch from a main feed artery. The embryonic and fetal vasculature is highly plastic and many vessel segments are pruned, thus not all side-branches generated through sprouting or intussusceptive angiogenesis, will result in a patent arteriole or venule. Hemodynamic factors play a significant role in the remodeling of embryonic/fetal vascular networks, and at bifurcations adaptation to shear stress predicts the regression of the smallest side branches. Therefore, next to understanding formation of vessels, insight into the mechanisms that prevent pruning is needed. For example, if collateral arterial connections are detectable at birth, but pruned during subsequent stages of life, either evoked by local hemodynamic adaptations or triggered by cardiovascular risk factors such a smoking, therapeutic strategies could aim at preventing regression of such arteriolar connection, perhaps by intensive shear stress training (see chapter by Buschmann).

Next to the genetic factors, environmental factors may influence collateral arterial network design before birth. Epidemiological and experimental studies have shown an association between low birth weight and cardiovascular diseases later in life [49]. These studies suggest that exposing a developing fetus to adverse conditions *in utero*, for example fetal hypoxia or malnutrition, results in long-lasting structural and functional changes in the cardiovascular system predisposing for ischemic cardiovascular diseases [50]. Exposure to hypoxia affects fetal cardiac pump function, and organ perfusion. Given the important role for blood flow in controlling the design of the arteriolar architecture, such hypoxic stress insults, via changes in cardiac output and arterial flow, may modulate preexisting collateral number, thereby predisposing for ischemic cardiovascular diseases.

Taken together: branched arterial networks are generated during embryonic development controlled by myriad genetic networks and hemodynamics. In the context of collateral arteriogenesis, two aspects from analyzing embryo development surfaced that may be relevant for novel therapeutic interventions: a) understanding the molecular growth control mechanisms determining preexisting collaterals, b) preventing the pruning of potential collateral connections that were generated during early life. The future challenge is to identify the full repertoire of molecules involved in the design of the arteriolar architecture, and collateral artery development.

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Chapter 3. Notch Signaling in Arteries: From Embryonic Development to Postnatal Homeostasis and Growth

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Florian P. Limbourg

Abstract: Development and adaptation of arteries are controlled by genetic programs and environmental cues. The evolutionary conserved Notch signaling pathway plays a major role in a distinct arterial gene program which fashions the unique features of the arterial system. Notch ligands and receptors are involved in many different aspects of arterial development and growth. These include the specification of arterial identity, regulation of sprouting angiogenesis, differentiation of arterial smooth muscle cells, arterial wall assembly and postnatal arteriogenesis. However many aspects of the molecular mechanisms remain unexplained. The present chapter aims to summarize the current knowledge about Notch signaling in development, adaptation and disease of arteries. Expression patterns of Notch ligands and receptors in the vascular system will be reviewed and phenotypes of general and conditional Notch pathway mutants will be discussed. Finally, to provide perspectives for future research, this chapter will raise some unanswered questions how Notch controls arterial growth and homeostasis.

Introduction

The vascular system of all mammalian and most non-mammalian species is a highly diversified hierarchical tree of differently sized tubes. These tubes share a single common lumen whose wall is made up from endothelial cells (EC), which set up a morphological and physiological barrier between flowing blood and the surrounding tissue. The embryonic *de novo* development of immature vessels from angiogenic blood islands is termed vasculogenesis [1,2]. The resulting first primitive vascular network consists only of EC. Outgrowth of capillaries is called sprouting angiogenesis, a process which is initiated by specialized EC and takes place both in embryonic development and in postnatal life. The immature vascular network in the embryo further differentiates into arteries and veins, which was thought to be driven by the onset of blood flow by the beating heart [3,4]. However, a number of seminal studies changed this paradigm by demonstrating that the differentiation of arteries and veins is regulated by a molecular program, which is activated independent of blood flow [1,5,6]. This molecular program is controlled by the evolutionary highly conserved Notch signaling pathway, which is instructive for arterial differentiation.

Development and postnatal growth of arteries share many morphological and molecular features. Both development and growth comprise the proliferation and temporo-spatial assembly of EC, smooth muscle cells (SMC), and in some cases pericytes to build or to enlarge an arterial vessel. Arteriogenesis, i.e. the growth of postnatal collateral arteries to conductance vessels, recapitulates many molecular and functional aspects of arterial development. Here we summarize the role of the Notch signaling pathway for each arterial cell type in embryonic development and postnatal life. Additional information is provided in tables summarizing markers of EC and SMC identity, regulators of Notch ligands, Expression of Notch pathway members and phenotype of mutant mice. Understanding the numerous aspects of Notch signaling in the embryonic arterial system is both essential and suggestive for fully discovering its role for postnatal adaptation and growth.

The Notch signaling pathway

The Notch signaling pathway is an evolutionary conserved intercellular signaling pathway. It plays several critical roles during development and physiology, primarily in the vascular system [7,8], the nervous system [9] and hematopoiesis [10-12], but also for skin, liver and gut development. In general, the Notch signaling pathway can act through diverse mechanisms, e.g. making binary cell-fate decisions, controlling boundary formation and

regulating cellular proliferation or differentiation. In mammals it consists of four transmembrane receptors (Notch1 through Notch4) and five ligands (Delta-like 1/-3/-4, Jagged1, Jagged2). Unlike in most other signaling pathways ligands of the Notch family are present only at the cell surface and not known to be secreted. Thus Notch signaling depends on an intimate cell-cell contact to allow ligand-receptor binding, which restricts it to signaling between neighboring cells. This type of action is also known as juxtacrine signaling. Upon binding of the ligand the receptor protein is proteolytically cleaved by membrane-tethered enzymes of the ADAM metalloprotease family and the gamma-secretase complex (Fig. **1**) [13]. Cleavage results in the release of the intracellular domain of the Notch receptor (NICD) into the cytoplasm. NICD then shuttles into the nucleus because of the presence of nuclear localization signals. Here it binds to recombination signal binding protein for immunoglobulin kappa J region (RBPjκ)[1], a sequence-specific DNA-binding protein. Upon binding of NICD, RBPjκ converts from a transcriptional repressor to a transcriptional activator inducing Notch target gene expression. Known Notch target genes are the basic helix-loop-helix (bHLH) transcriptional repressors of the hairy and enhancer of split (*Hes1*, *Hes5*) and hairy and enhancer of split related with YRPW motif (*Hey1*, *Hey2*) family [14].

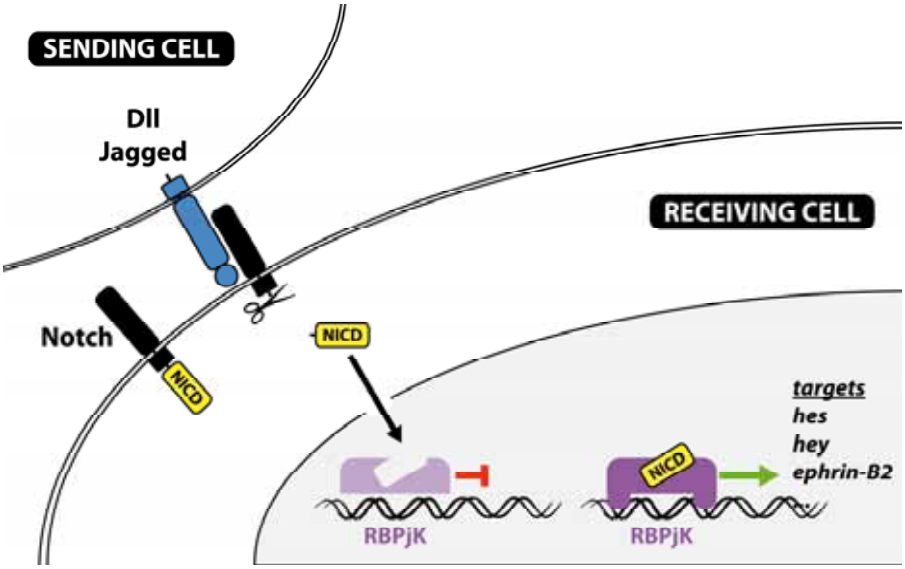

Figure 1: Overview of the Notch signaling pathway. Ligand-bearing cells activate Notch in adjacent cells by binding the receptor protein, which requires intimate cell-cell contact. This mode of signaling is also called ‚juxtacrine'. After cleavage of the receptor its intracellular domain (NICD) translocates into the nucleus, binds to RBPjκ and leads to target gene expression.

This signaling cascade is termed the canonical Notch signaling pathway. However, there is some evidence for non-canonical Notch signaling, i.e. signaling without involvement of RBPjκ [15]. Further details on the intracellular signaling mechanisms can be found in several recent reviews [13,14,16-18]. Upon receptor binding the ligand is internalized by the sender cell. Ligand internalization may also exert specific effects in the sender cell known as reverse signaling [13,19], since modification of the cytoplasmic domain of the ligand causes a phenotype [20].

Notch usually signals *in trans*, i.e. the ligand bearing cell activates Notch in an adjacent cell. In some cases the same cell carries both the ligand and the receptor, e.g. arterial EC. Notch

[1] RBPjκ is also named C-promoter binding factor 1 (CBF1) in mammals and suppressor of hairless [Su(H)] in Drosophila melanogaster.

signaling *in cis*, i.e. one cell is sender and receiver at the same time, also takes place [21,22]. In contrast to *trans* signaling this process is believed to be inhibitory [18,23,24]. However, whether there is a significant role for *cis* [18] signaling *in vivo* is poorly understood.

Compelling evidence suggests that every action of Notch depends on a specific ligand-receptor pair in a given temporo-spatial setting and that such pairs exert specific effects in a given context. Even if the general role of Notch in vascular development and disease has been well established, exact roles of specific ligand-receptor pairs remain to be characterized [8].

Embryonic arteriogenesis

Arterial identity is derived from a predefined molecular program in EC (Fig. **2**), which precedes establishment of mature arteries. In embryonic development, a primitive endothelial network is formed in situ [25-28]. This primitive tubular network is then remodeled into a highly diversified and complex network of differently sized arteries, capillaries and veins. The fully developed vascular system has a distinct anatomy on every step of the vascular tree [29]. The aorta is the biggest artery whith a strong wall containing a thick layer of smooth muscle cells, which gets thinner the smaller the artery becomes. Blood flow, blood pressure and oxygen tension change throughout the vascular tree. After the onset of the circulation the morphological and functional diversity of the vascular tree becomes more and more apparent. Thus, it was long believed that hemodynamic forces, starting as the heart begins to beat, drive the diversification of the immature and primitive vascular network into a complex system of arteries and veins [3,4]. However, pivotal studies on vascular identity before onset of the first heart beat demonstrated that distinct genetic programs precede blood flow, challenging the paradigm that flow determines that decision [6,30,31].

The developing zebrafish embryo allows detailed investigation of vascular formation [32]. Zebrafish are translucent and several transgenic lines expressing fluorescent proteins in different cell types are available. This makes this model organism an ideal system to perform real time analysis of the growing vasculature, mostly done by time lapse video microscopy. In the developing zebrafish two main vessels form early after gastrulation: The dorsal aorta and the posterior cardinal vein. The study of the formation of these two vessels offers a comfortable opportunity to study arteriovenous differentiation. The molecular difference between arterial and venous EC was first shown by the identification of ephrin-B2 as an artery-specific endothelial ligand and EphB4 as a venous-specific receptor [6,33,34]. Lineage tracing in zebrafish embryos revealed that the hemangioblast is molecularly dedicated to give rise to either arterial or venous EC but not both [30]. Notch is upstream of ephrin-B2 and required for arterial identity and dorsal aorta formation [30,35-37]. Notch mutant embryos show an impaired formation of the dorsal aorta and the posterior cardinal vein and exhibit fusion of the two vascular beds without intervening capillaries. In these embryos arterial markers such as ephrin-B2 are lost and venous markers like EphB4 are gained, indicating defective arteriovenous differentiation. The Notch target gene seems to mediate arterial differentiation signals: Targeting *gridlock* in zebrafish leads to a disruption of dorsal aorta formation [30,35].

Mutant phenotypes were also extensively studied in mice. The developing yolk sac of mice demonstrates all stages of vascular development: vasculogenesis, angiogenesis and arteriogenesis. In yolk sacs of Notch1$^{-/-}$ mice a primitive plexus forms which lacks any further remodeling, indicating that stages above vasculogenesis require Notch1 signaling [38]. The phenotype of global Notch1 mutant mice is mainly due to a loss of Notch1 in the vascular endothelium. Ablation of Notch1 specifically in EC with a Tie2-driven Cre deleter in conditional Notch1$^{flox/flox}$ mice fully recapitulates the global Notch1$^{-/-}$ phenotype [39].

A direct link in mice from canonical Notch signaling to arterial identity was made by using RBPjκ$^{-/-}$ mice which carry a *lacZ* transgene under the control of the *ephrin-B2* promoter. The yolk sac of these mice lacks all remodeling beyond initial vasculogenesis. It does not show any expression of ephrin-B2 compared to a strong signal in RBPjκ$^{+/+}$ mice carrying the same transgene [40]. These data confirm that canonical Notch signaling is required for plexus maturation and arterial cell fate specification.

Studies in the developing zebrafish embryo further uncovered the signaling cascade responsible for arterial differentiation. Expression of vascular endothelial growth factor (VEGF) is regulated by the secreted morphogen sonic hedgehog (shh) along the axial midline [41]. Shh is necessary for arterial identity, and overexpression of Shh induces ectopic arterial marker expression in the cardinal vein [41]. Loss of Shh can be rescued by *vegfa* mRNA injection, and *vegfa* overexpression again induces arterial markers in the cardinal vein. *Vegfa* mutants exhibit the same phenotype as *Shh* mutants which can be rescued by a *Notch1* transgene [41]. These results showed that Shh, VEGF-A and Notch1 act in sequence during arterial differentiation in developing vessels.

Knockout mice lacking *ephrin-B2* either in the whole body or specifically in EC showed a similar defect like Notch1 mutants: Formation of the primitive vascular network occured, but differentiation into hierarchical networks of vessels of different size and identity was missing [6,42]. As Notch directly regulates ephrin-B2 [41,43,44] and the knockout phenotypes of Notch1 and ephrin-B2 are highly similar, it is now clear that Notch acts upstream of ephrin-B2 in the arterial differentiation cascade.

EphB4, the receptor for ephrin-B2, is a specific marker for venous EC. Interstingly, loss of either ephrin-B2 or EphB4 leads to a similar embryonically lethal phenotype lacking arteriovenous differentiation [6,33,34]. This demonstrates that bidirectional signaling between ephrin-B2 and EphB4 determines EC fate, which in turn is controlled by canonical Notch signaling.

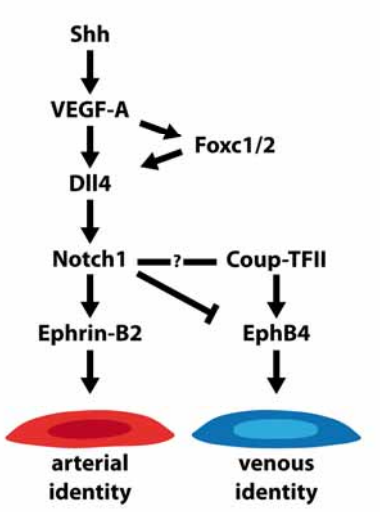

Figure 2: The molecular cascade which controls endothelial differentiation during vascular development. Dll4-mediated Notch signaling both determines arterial identity and inhibits venous differentiation.

Expanding the results from mice and zebrafish recent studies have placed the Notch ligand Dll4 into this signaling cascade. VEGF-A regulates Dll4 through VEGF-Receptors 1 and -2 and the phosphatidylinositol 3-kinase/Akt pathway [45]. Dll4-deficient embryos as well as RBPjκ mutants and Hey1/Hey2 double-mutant embryos do not express arterial markers [40,46-49]. Dll4-mediated Notch signaling induces ephrin-B2 expression in EC *in vitro* [50] and the *ephrin-B2* gene has recently been shown to be a direct Notch target gene [44], demonstrating a direct and instructive role for Dll4-mediated Notch signaling in arterial differentiation.

Mutant mice which lack Foxc1 and Foxc2, transcription factors of the *forkhead* family, show severe defects in the developing yolk sac and the embryo proper [51], mimicking the Dll4 or Notch1 knockout phenotype. These mice also exhibit accompanying AV-malformations. Foxc1 and Foxc2 directly activate *dll4* and *hey2* transcription in EC by binding to the respective promoters [52,53], which is enhanced by VEGF-A. Thus there is strong evidence that Foxc1 and Foxc2 are directly involved in arterial specification of the developing vasculature by regulating Dll4. These findings further extend the signaling network responsible for arterial specification of EC.

Whether adoption of arterial or venous cell fate is irreversible has remained an important question since it has implications for understanding the mechanisms of vascular differentiation and regeneration. Insights into EC plasticity came from transplantation experiments: EC from transplanted embryonic quail arteries or veins colonize both recipient arteries and veins in the developing chick embryo, suggesting some EC plasticity. However this is only the case at specific stages of embryonic development: Grafts isolated from quail embryos after E7 progressively lose plasticity, and EC grafted after E11 only colonize vessels from which they originated [54], indicating that EC fate is fixed at late stages of development. However, experiments in chicken embryos during early phases of development demonstrated that hemodynamic forces are sufficient to revert EC identity, supporting that EC plasticity is

present in early development [55]. Notch signaling determines the homing behaviour of transplanted EC in zebrafish: EC in which Notch1 is overexpressed integrate into arteries. On the other hand, RBPjκ deficient EC integrate into veins, indicating predefined identity and homing of EC, but also demonstrating that Notch signaling specifies cell fate cell-autonomously in this context [56]. A very recent study found that coronary arteries develop from venous endothelial cells [57], which likely reflects that cardiac arteriogenesis is special and differs from vascular development in other territories. Irreversibility of differentiated EC identity can also be observed in humans: Veins used as bypass homografts between the aorta and the coronary arteries on human hearts morphologically adapt to the new flow conditions but do not gain full arterial identity, which underlines the inherited molecular identity of a given vessel [58]. Taken together EC plasticity seems to be present at early developmental stages, but fully established EC identity in a developed vascular system is irreversible.

Many experimental findings demonstrate that Notch signaling is instructive for arterial differentiation and at the same time suppresses venous fate. This led to a paradigm that an undifferentiated endothelial cell adopts arterial identity once Notch is activated or aquires venous fate by default. However venous fate is regulated by chicken ovalbumin upstream promoter–transcription factor II (COUP-TFII) [59]. COUP-TFII suppresses Neuropilin-1 expression which is important for the arterial differentiation cascade downstream of VEGF-A. In COUP-TFII mutant mice venous EC adopt arterial markers but still express considerable amounts of EphB4, suggesting incomplete arterial differentiation. On the other hand, arterial vessels of transgenic mice overexpressing COUP-TFII have completely lost arterial identity and adopt venous fate [59]. These findings further expand the current knowledge about arteriovenous differentiation from a vertical cascade to a complex system which regulates endothelial identity.

However, given the pleiotropic role of VEGF for the vascular system it is not surprising that VEGF alone is not sufficient to determine arterial identity. VEGF120, a soluble VEGF isoform, is expressed by peripheral nerves and contributes to local arteriogenesis [60]. However venules are close by, are also exposed to locally produced VEGF and express the same high affinity VEGF receptors. But they do not express arterial markers, confirming that arterial identity is a predefined specificity and that VEGF alone is not sufficient for its determination. It is tempting to speculate that the combination of VEGF coreceptors like Neuropilin-1 and expression of Notch receptors may result in a general responsiveness of EC to different VEGF isoforms for determining arterial identity [61].

Sprouting angiogenesis

During angiogenesis new vessels form from existing ones by capillary sprouting [62]. Specialized EC at the tip of the outgrowing sprout (‚tip cells') guide the growing vessels along environmental cues [63-65]. Adjacent proliferating EC (‚stalk cells') trailing behind the tip cell form the trunk of the new sprout.

Two popular animal models exist for the investigation of sprouting angiogenesis: The formation of intersomitic vessels in the zebrafish embryo and vascularization in the retina of newborn mice. The retinal vascular system develops shortly after birth before opening of the eyes. The first vessels emerge at the insertion of the optic nerve in the retina centre, building an angiogenic front. This front migrates radially into the avascular zone towards the ora serrata. A gradient of matrix-bound VEGF, which is produced by local astrocytes, serves as a guidance cue for this migration process. The cells at the leading edge are specialized migrating tip cells extending numerous cellular filopodia which sense the VEGF gradient. All EC following the angiogenic front are called stalk cells, are highly proliferative and mainly build the vascular sprout. In this, EC recapitulate a general growth mechanism which is also utilized by other cellular networks, e.g. outgrowing neurons and the developing trachea [66-68]. The combination of tip cell migration and stalk cell proliferation results in vascularization of the whole retina by a tubular plexus. In a second step this primitive vascular plexus is remodeled into large and small arterial and venous vessels. At all times the retinal vasculature is easily accessible both for investigation and manipulation, making the mouse retina model a very good model for studying vascular growth and postnatal arterial development.

Until recently the molecular mechanisms of tip and stalk cell selection and EC guidance were largely unknown. Some years ago a role for Notch signaling in tip cell physiology was first described [69]. In an *in vitro* system using human umbilical vein EC, inhibition of Notch signaling led to a tip cell phenotype and cell division, leading to two daughter tip cells. Notch activation inhibited sprout formation, while Notch inhibition enhanced it by increasing the number of tip cells and tip cell filopodia.

This initial discovery was confirmed by recent landmark studies which independently discovered a general mechanism of Dll4-Notch1 signaling in VEGF induced sprouting [70]. The central findings of these studies were made in different model systems: the mouse retina and hindbrain [71-74], the developing zebrafish [56,75] and xenograft tumor models [74,76,77]. Dll4 is strongly expressed in tip cells due to VEGF-A stimulation from the local miroenvironment. Dll4 in turn activates Notch1 in adjacent stalk cells, which leads to downregulation of VEGF-R2. This results in decreased susceptibility of stalk cells to VEGF, which hinders the cell from becoming a tip cell. On the other hand, once Notch signaling is inhibited the number of tip cells and filopodia is excessively increased resulting in more sprouting. These studies further confirm that VEGF is upstream of Notch, because inhibition of VEGF reduces Dll4 expression, thereby controlling sprouting angiogenesis and vascular guidance [75]. In summary, Dll4 from tip cells inhibits sprouting angiogenesis by suppressing tip cell differentiation in neighbouring EC.

However, vascular development in the mouse retina can only be investigated statically. This gap is elegantly filled by the analysis of intersomitic vessel formation in zebrafish. Similar to astrocytes in the mouse retina, somites produce a gradient of VEGF which is sensed by tip cell filopodia. It was found that the same mechanisms as in the mouse retina control intersomitic vessel formation: Upon VEGF exposure tip cells express Dll4, which activates Notch1 and downregulates VEGFR-2 in stalk cells, thereby hindering the stalk cell from becoming a tip cell [56,75].

Vascular sprouts in Dll4 and RBPjκ morphant zebrafish embryos contain more EC compared to wildtype embryos due to increased recruitment of EC to the growing vascular sprout and increased proliferation of EC within the sprout. This indicates the Dll4/Notch1 signaling suppresses proliferation of stalk cells. However these studies also showed that the RBPjκ phenotype is more severe than the Dll4 phenotype, suggesting that other Notch ligands play an important role [56,75].

The question how the complex patterning of the developing vascular system is modulated by different Notch ligands was elegantly answered by a recent pioneer study demonstrating opposing roles of Dll4 and Jagged1 in sprouting angiogenesis [78]. Jagged1 is strongly expressed in stalk cells during retinal angiogenesis. Mice which lack Jagged1 selectively in the endothelium show reduced vascularization and fewer tip cells with fewer filopodia in the angiogenic front. At the same time proliferation of EC is reduced when endothelial Jagged1 is lost. On the other hand Jagged1 overexpression in the endothelium leads to the opposite phenotype: Tip cell and filopodia numbers are increased and EC proliferation is enhanced resulting in augmented retinal angiogenesis. Since activation of Notch1 by Dll4 suppresses sprouting, how can Jagged1 promote angiogenesis? The study found that Jagged1 and Dll4 are not only expressed in a complementary fashion, but that Notch target genes like Hes1 and Hey1 are induced when Jagged1 is lost. During sprouting angiogenesis Jagged1 therefore seems to be an inhibitory Notch ligand, which competes with Dll4. This led to a model in which two Notch ligands hold a balance of Notch activation resulting in tightly controlled angiogenesis.

The glycosyltransferase *fringe* modulates Notch activation upon exposure to different ligands by posttranslational modification of the receptor protein [78]. Fringe enhances Dll4-mediated and reduces Jagged1-mediated Notch activation. These findings add another level of regulation beyond the distribution and subtypes of Notch ligands and receptors.

The angiogenic response is mediated by VEGF receptors. Tip cells express VEGF-R3 and stalk cells VEGF-R2. This balance is supposed to be regulated by opposing actions of Dll4 and Jagged1 [78]. Indeed, inhibition of VEGF-R3 by genetic targeting or with monoclonal antibodies blocks sprouting angiogenesis [79].

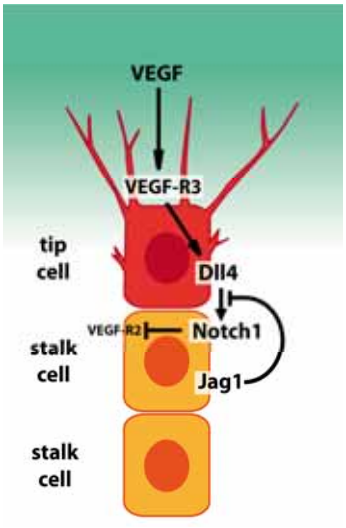

Figure 3: Dll4 and Jagged1 control angiogenic sprouting by balancing endothelial Notch activity.

In summary, all findings so far show that the Notch ligands Dll4 and Jagged1 control sprouting angiogenesis by balanced Notch activation, which regulates tip and stalk cell selection and their function by the regulation of VEGF receptors 2 and 3.

However, important aspects of sprouting angiogenesis still remain to be explained. First, increased sprouting and branching results from inhibition of the Dll4/Notch axis at different levels, such as in $Dll4^{+/-}$ mice, by deleting Notch1 in EC or by pharmacological Notch inhibition. These phenotypes are similar in the gross view, but fine differences are evident: pharmacological Notch inhibition, which inhibits activation of all Notch receptors, increases sprouting only at places of active angiogenic migration. This leads to the formation of vascular syncytia. These syncytia strongly resemble AV malformations observed in Notch1 mutant zebrafish and mouse embryos (*see below*). This confirms that dysbalanced endothelial Notch signaling impairs vascular boundary formation. In Dll4 mutant mice filopodia protrusion is increased in the whole retina but syncytia do not form to this extent. The fact that the phenotypes observed by different ways of Notch inhibition slightly vary suggests that the mechanisms of angiogenesis in this context are still not fully discovered.

Second, stalk cells are thought to be highly proliferating cells to build the vascular trunk. Dll4 mediated Notch activation is known to suppress proliferation which stands in contrast to this. Maybe the initial paradigm of migrating tip cells on one hand and proliferating stalk cells on the other hand can not be maintained. The studies in zebrafish reveal that the tip cell at the leading edge undergoes cell division and leaves one daughter cell behind [56]. This would favour a model in which tip cells are asymmetrically dividing EC leading the sprout and forming the trunk at the same time.

Third, it is still unclear how a cell becomes a tip cell, since it is believed that all EC ‚start' with the same molecular equipment. Initially it was thought that only the cell that experiences the highest VEGF concentration becomes a tip cell, i.e. tip cell differentiation would be a stochastic event. However this might not fully explain the mechanism [71]. The VEGF gradient is believed to guide tip cells radially towards the ora serrata. Vascular growth and subsequent perfusion reduce local VEGF expression through astrocyte differentiation. But how do side branches form? Can a stalk cell become a secondary tip cell to found a side branch, and is there a role for hemodynamic forces? After sprouting angiogenesis has covered the retinal surface vertical branching seeds a deep vascular plexus [78]. How is vertical branching regulated? It is in several aspects different from sprouting: It occurs from an established vascular plexus and not in parallel to vascular sprouting despite VEGF is present. Sprouting EC on the retinal surface do not need to migrate through tissue layers in contrast to branching EC. Taken together the current concept of sprouting angiogenesis does not explain vertical branching and three-dimensional vascular network formation.

At last stalk cell identity and function are not well understood. If VEGF determines tip cell identity, then in the next step Dll4 would suppress tip cell identity in the neighbouring EC, resulting in a stalk cell. But what happens to the stalk cell behind the stalk cell? Do stalk cells constitute a homogenous population of EC? It is still unclear whether Jagged1, even if it is expressed in most stalk cells, determines stalk identity in following EC.

Notch signaling also plays an important role in tumour angiogenesis, which is in many aspects similar to physiological sprouting. Tumour growth critically depends on oxygenation, and most tumours induce pathological angiogenesis for their own survival [80]. Many human solid tumours therefore express large amounts of VEGF and also the Notch ligand Dll4 at high levels [47]. Anti-VEGF antibodies are approved for treatment of many human tumours,

and novel therapies targeting Dll4 have been shown to reduce tumour growth. As we know from retina and zebrafish studies inhibition of Dll4 leads to more sprouting and branching, and this also happens in murine xenograft tumour models. Interestingly, tumour growth is reduced when Dll4 is targeted [74,76,77]. The vasculature in such treated tumours shows more capillaries due to increased sprouting and branching, however the capillaries seem to function not effectively as the vascular network is not set up properly and many capillaries are not connected to the network. As a result the tumours are less well perfused than control tumours leading to impaired tumour growth: Increased capillarization – less perfusion. Even if many more trials need to be performed before anti-Dll4 therapies reach the clinic, this is a promising new therapeutic approach [81]. However, from a physiological point of view this underlines the essential role of a well diversified arterial network for sufficient organ perfusion. Thus angiogenesis alone is not enough and might also have adverse effects when considering new approaches for regenerative therapies.

Jagged1 has also been shown to be expressed in tumours and to enhance neovascularization and tumour growth. In contrast to anti-Dll4 therapies a soluble Notch1 decoy receptor led to reduced tumour growth without increased vascularization. This indicates that the principle of ligand balance for controlling sprouting angiogenesis [78] might also hold true for tumour angiogenesis, but that roles of individual Notch receptors would be different.

AV malformations occur when arterial identity is lost

Normally, the arterial and venous system is connected by capillaries, and formation of this three step system depends on the specification of arterial and venous identity. Once this is lost, AV malformations form which indicates disturbed vascular identity. In loss of function Notch mutants, e.g. Notch1$^{-/-}$ or Dll4$^{-/-}$, AV-malformations are readily detectable [40,46,47]. Interestingly AV malformations also occur in Ephrin-B2 and in EphB4 deficient mice [6,33], indicating that perturbation of the arterial differentiation cascade at any step leads to a defective vascular network.

Notch1$^{-/-}$ embryos die in utero from severe vascular defects. Mice homozygous for a null mutation of the *Notch4* gene do not exhibit an obvious mutant phenotype, presumably because of functional redundancy with the *Notch1* gene [38]. However, Notch1/Notch4 double mutants show a more severe vascular phenotype compared to Notch1$^{-/-}$ embryos, indicating that Notch1 and Notch4 are closely interrelated in a complex endothelial signaling network.

At first glance, one could assume that Notch signaling functions as an on-off switch: Notch on – arterial identity, Notch off – default venous cell fate. However, Notch signaling needs to be finely tuned and precisely timed. For example, overexpression of Notch4 in EC leads to premature death at embryonic day E10 due to defective embryonic vasculature [82]. The phenotype of these mutants is very similar to the Notch1/Notch4 double knockout [38]. The similarity between a gain-of function and loss-of-function mutation in the same gene family demonstrates that appropriate and balanced levels of Notch signaling are critical for proper vascular development. AV malformations also occur in humans and altered Notch signaling has been detected in human brain AV malformations [83,84]. Furthermore, conditional overexpression of Notch4 in postnatal EC leads to AV malformations in mice [84,85], strongly suggesting that dysbalanced Notch signaling plays a causative role also in human AV malformations.

Notch and vascular smooth muscle cell homeostasis

Most studies on the role of Notch in the vascular system focused on EC in development and physiology. However Notch signaling is not only a master regulator of endothelial cell fate and function, but also plays a critical role for vascular SMC. SMC in human adults are not terminally differentiated but partially retain plasticity to respond to external cues. Consequently SMC shift between proliferation, synthesis and differentiation. When a vessel grows SMC change their phenotype from differentiation to a synthetic and proliferative

phenotype. Thus, genes regulating the SMC phenotype are of fundamental interest for understanding vascular growth.

Initial reports suggested that Notch *suppresses* SMC differentiation *in vitro* via induction of Hey2 [86-88]. The exposure of SMC to cyclic mechanical strain represses Notch1 and Notch3 expression and induces SMC differentiation markers [89,90]. These cells also show reduced proliferation and increased apoptosis rates. Overexpression of Notch1 or Notch3 intracellular domains in these cells restored normal levels of proliferation and apoptosis, suggesting that the effects of hemodynamic forces on SMC phenotype shifts are at least in part regulated via Notch. Neointima formation by vascular SMC is significantly impaired in $Hey2^{-/-}$ mice [91], and aortic SMC isolated from $Hey2^{-/-}$ mice show reduced proliferation compared to those from wildtype controls. SMC overexpressing Hey1 [92] or Hey2 [93] show higher proliferation rates with reduced levels of the cyclin-dependent kinase inhibitors p21 and p27 respectively. Hey2 seems to be a direct transcriptional repressor of $p27^{kip1}$ [93]. However, canonical Notch signaling activated by Jagged1 *induces* SMC differentiation [94,95]. Marker genes for SMC differentiation such as smooth muscle myosin heavy chain (SMMHC) [94] and smooth muscle alpha-actin (SMA) [96] were identified as direct Notch target genes.

Mice in which Notch signaling is completely ablated specifically in vascular SMC are viable and fertile. They show vascular abnormalities in cerebrovascular patterning. After carotid ligation these mice die from stroke due to insufficient collateral blood flow in the circle of Willis [97]. This indicates that Notch in vascular SMC is not necessary for general vascular development but that detailed arterial patterning, and possibly adaptation to injury requires cell-autonomous Notch signaling in SMC. This is confirmed by data from Notch3 mutant mice. Notch3 is specifically expressed in arterial but not in venous SMC [98]. $Notch3^{-/-}$ mice are also viable and fertile but show severe arterial defects with dilated arteries and a thinned SMC layer compared to wildtype mice [99,100]. There are just few markers for arterial identity in SMC, such as smoothelin [101] and SM22a [102]. Interestingly, SMC of $Notch3^{-/-}$ mutant mice have drastically reduced expression of these markers, suggesting together with morphological differences that these SMC have lost arterial identity. However, while SMC lose arterial identity in $Notch3^{-/-}$ mutant mice, EC do not: They still express all established arterial EC markers such as ephrin-B2 and *Hes/Hey* genes, demonstrating that loss of SMC arterial identity does not influence EC identity [99].

Regarding the results of experiments on Notch signaling in SMC it is likely that Notch acts context-dependent. One very remarkable feature of Notch signaling is that ligand-receptor interaction results in different effects depending on the context. This is mainly due to the fact that Notch signaling depends on intimate cell-cell contact. Thus, the following questions are essential: Which cell bears the ligand and which one the receptor? Which ligand and which receptor are present? Is more than one ligand involved? At which step of a given process will the signaling event take place? Most of these questions are not answered yet for vascular Notch signaling. This is especially important since many cells in the cardiovascular system carry both the ligand and the receptor. It is therefore critical to perform studies in mice with concise conditional knockout concepts to find out in which cell type under which condition a given member of the Notch family is important.

Usually Notch signals *in trans* between neighboring cells. If these two cells are of the same type it is called a homotypic interaction, if not it is called a heterotypic interaction. However, Notch can also signal *in cis*, i.e. a ligand on a given cell interacts with a receptor on the same cell which is believed to be inhibitory [23,24]. In general, Notch signaling inside the arterial wall *in vivo* would occur between neighboring EC (trans, homotypic), from EC by filopodia through the basal membrane to SMC (trans, heterotypic) or from SMC to SMC (trans, homotypic) [103]. Regarding the expression profile of Notch ligands and receptors in the adult arterial wall (Fig. **5**, Table 3), it is tempting to assume that such interactions take place, however very little is known about these processes. It has been shown that Jagged1 from EC activates Notch3 in SMC *in vitro* [104]. Deleting Jagged1 selectively in the endothelium recapitulates the global $Jagged1^{-/-}$ phenotype: Mutant embryos die around E10.5 from severe cardiovascular defects. Interestingly Notch1 activation and ephrin-B2 expression in the arterial endothelium were normal, suggesting that endothelial Jagged1 does not signal in trans to EC and that loss of arterial identity in the endothelium does not account for the phenotype. However markers of arterial differentiation like SMA and SM22a were not expressed in SMC

of mutant embryos anymore, indicating that loss of Jagged1 in the endothelium disturbs SMC differentiation [105]. These results would favour a model of trans heterotypic signaling from EC to SMC. Whether this happens directly or indirectly remains unclear. Future studies employing distinct cell-type specific gene targeting will hopefully decipher the exact role of Notch signaling for SMC homeostasis and growth.

CADASIL

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited disease which is thought to be caused by a mutation in the *Notch3* gene. It is the most common genetic cause of stroke and vascular dementia in adults [106].

CADASIL patients suffer from various symptoms including mood disorders, cognitive decline, headaches, dementia or recurrent strokes, often resulting in premature death. Histopathological findings include degeneration of vascular SMC and accumulation of granular osmiophilic material (GOM) at the SMC basement membrane [107]. The accumulation is a typical and characteristic feature of CADASIL biopsy results.

The mutations found in the *Notch3* gene are located in the EGF-like repeats of the Notch3 extracellular domain and do not lead to inactivation of the receptor. The mutations rather lead to accumulation of the Notch3 ectodomain in the cerebral microvasculature and constitute parts of the GOM deposition. Two mouse models of CADASIL exist, both of which carry *Notch3* mutations found in patients. In one model, the endogenous murine *Notch3* gene carries an Arg142Cys knock-in mutation [108]. However, these mice do not show CADASIL-like symptoms or a related phenotype. The second model is a conditional mutant mouse carrying a human *Notch3* cDNA with the Arg90Cys mutation in vascular smooth muscle cells [109]. These mice show classical histopathological features like Notch3 ectodomain accumulation and GOM deposits in arterioles, however a parenchymal brain damage is lacking. The cerebral vasoreactivity in these mice is impaired, suggesting that the introduced *Notch3* mutation affects arterial function [110]. On the other hand, the introduced mutation does not impair Notch3 signaling *in vivo* [111]. Taken together, CADASIL seems not to be caused by impaired Notch3 signaling but by a novel mechanism which leads to Notch3 protein accumulation. Whether the accumulated Notch3 protein acts as a decoy and locally inhibits Notch signaling or unspecifically disturbs SMC integrity remains unclear. Further insights from existing or novel mouse models will hopefully completely explain the disease mechanisms and lead to new therapeutic approaches in the future.

Notch signaling and postnatal arteriogenesis

The rescue of ischemic tissue critically depends on adequate perfusion. In postnatal life arteriogenesis, i.e. the growth of collateral arteries to conductance vessels, is sufficient to restore perfusion after disruption of blood flow (Fig. **4**). To date much more is known about specific roles of Notch signaling for embryonic than for postnatal arteriogenesis. However the indispensable role of Notch signaling for both embryonic development of arteries and homeostasis of the arterial system in later life strongly suggest that Notch pathway members control postnatal arterial growth. Consequently very recent pivotal studies demonstrate an essential role of Notch signaling for postnatal arteriogenesis [43,112,113].

Notch1$^{-/-}$ mice die during embryogenesis from severe vascular defects, involving failure to remodel the primary vascular plexus [38]. Endothelial Notch1 ablation fully recapitulates this phenotype [39]. However global and endothelial-specific Notch1 heterozygous mice are viable. Limb necrosis after ischemia is strongly increased in these mice and blood flow recovery is impaired while VEGF-A upregulation is not altered compared to control mice [114]. This indicates that endothelial Notch1 is important for postnatal arterial growth. Furthermore this demonstrates that a genetic program which controls embryonic arteriogenesis is also essential for postnatal arterial growth.

Postnatally the Notch ligand Dll1 is expressed selectively in the endothelium of medium and large arteries but not in veins or capillaries [43]. To test its role for postnatal arteriogenesis we employed a well established model of hind limb ischemia (Fig. 4), in which defined collateral

arteries in the mouse hind limb grow upon ligation of the femoral artery [115]. After ligation altered hemodynamic parameters such as fluid shear stress dynamically trigger initiation and maintenance of collateral arterial growth [116,117]. During growth of collateral arteries Dll1 is strongly upregulated in the endothelium, and Notch signaling is activated as shown by the appearance of cleaved Notch and upregulation of Hes1, Hey1 and ephrin-B2. Dll1 heterozygous mice are viable and fertile and do not show gross abnormalities of the vascular system. However, collateral arteries in these mice fail to grow upon femoral artery ligation, demonstrating that Dll1 is essential for postnatal arterial growth [43]. Limb necrosis and autoamputation is dramatically increased compared to wildtype controls. Induction of Notch target genes such as *Hes1*, *Hey1* and *ephrin-B2* in the vascular wall is lost in Dll1 heterozygous mice. Since Dll1 is specifically expressed in the arterial endothelium, these results suggest that endothelial Dll1 is a critical mediator of a growth signaling cascade and that Dll1 haploinsufficiency impedes vascular Notch signaling. Interestingly, VEGF was upregulated in wildtype as well as in Dll1 heterozygous arteries during growth, recapitulating that VEGF is upstream of Notch.

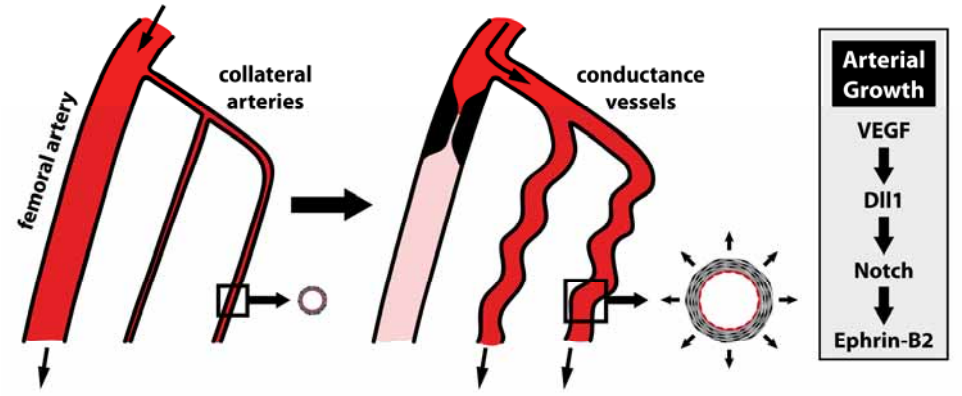

Figure 4: Postnatal Arteriogenesis. Occlusion of a main artery leads to growth of collateral vessels which become conductance arteries. Dll1-mediated Notch signaling in the vascular wall is essential for collateral artery growth and rescue of downstream ischemic tissue.

Another study confirmed the involvement of Dll1 in growth of postnatal arteries [112]. In this study a model of carotid ligation was employed, in which ligation of the left carotid artery leads to increased blood flow and outward growth of the unligated carotid artery on the right side. Dll1 was strongly induced in the unligated artery, and this induction happened to occur shortly after ligation, indicating that Dll1 expression might respond to changes in blood flow.

A very recent study investigated the vascular response to different flow conditions [113]. In human arterial endothelial cells pulsatile shear stress induced the expression of *Hey1*, *Hey2*, *Hes1* and *ephrin-B2*. Dll1 and Dll4 induction was not statistically significant but showed a strong trend. Another study reported induction of Notch1 and Notch4 mRNA in human umbilical vein endothelial cells exposed to cyclic strain [118]. Notch3, which is primarily expressed in arterial SMC, was recently identified to be important for vascular mechanotransduction. Resistance arteries from Notch3 deficient mice contract normally upon stimulation with agents like phenylephrine or angiotensin. However shear stress induced vasodilation is abolished in these mice, indicating that Notch3 in SMC plays a role in the arterial response to changes in the blood flow pattern [119]. Together these results suggest a link between shear stress as an indispensable regulator of arteriogenesis and vascular Notch signaling.

However, many questions are still unanswered: How is the spatial relationship between vascular wall cells regarding Notch signaling? Which receptors are activated during growth,

and which cells are the primary „receiver“ cells in this process? Is Notch signaling sufficient to induce arteriogenesis, maybe also in venous grafts? Ongoing studies will hopefully answer these and many more open questions in the near future.

Specific roles of individual Notch ligands in the vascular system

There is increasing evidence that given Notch ligand-receptor pairs exert specific effects, which suggests unique roles for individual ligands in the vasculature (Fig. **5**). The amount of ligand expressed on a given cell is important for the signaling process and should be considered when analyzing genetically engineered mice: Low expression might not be sufficient to induce Notch signaling consistent with a threshold model of receptor activation [120]. The cleaved part of the intracellular domain of the four Notch receptors is supposed to be identical in terms of the molecular structure, however this does not necessarily reflect functional identity. Transgenic mice expressing activated forms of Notch1 or Notch3 in thymocytes display different phenotypes, indicating that the intracellular domains of the four Notch receptors exert different functions downstream [121,122]. This assumption was not specifically tested yet in the vascular system, but is likely to hold true in this context as well.

Dll1

The expression of Dll4 precedes the expression of Dll1 in embryonic vessels [38,123,124], suggesting that the initial development of the arterial system depends on Dll4 and its maturation, diversification and growth might be controlled by Dll1. In EC-specific Dll1 mutants the expression of arterial markers like ephrinB2 is lost and venous markers like Coup-TFII are upregulated despite the continuing expression of Dll4 [124]. Dll1 mediated Notch1 activation upregulates VEGF-R2 (KDR) and its coreceptor neuropilin-1, suggesting that Dll1 enhances the responsiveness of an arterial endothelial cell to VEGF-A stimulation. $Dll1^{-/-}$ mice die from severe hemorrhage at E11.5 [125], i.e. slightly later than $Dll4^{-/-}$ mice, however $Dll^{+/-}$ mice are viable and fertile [43] in contrast to Dll4 heterozygous mice. After birth Dll1 is selectively expressed in the arterial endothelium and an essential ligand for postnatal arteriogenesis [43]. Taken together Dll1 is not required for vasculogenesis and the initial specification of arterial identity in EC, but essential for arterial maturation, maintenance of arterial identity and postnatal growth of arteries (Fig. **5**).

Dll3

$Dll3^{-/-}$ mice die in utero from severe skeletal defects due to disorganized somite formation [126]. Dll3 mutations in humans have been linked to a disease called spondylocostal dysostosis [127]. A role of Dll3 for the vascular system has not been reported yet (Fig. **5**).

Dll4

Dll4 is the first Notch ligand to be expressed in the developing vasculature [38,128,129]. It controls arterial identity and is indispensable for arterial network formation (Fig. **5**) [40,46,47]. As a result, $Dll4^{-/-}$ mice die at E10.5, and $Dll4^{+/-}$ are viable only under specific conditions (see Table 4). Dll4 expression decreases in the arterial endothelium during development and after birth Dll4 is detectable only in small but not in large arteries [43]. Whether Dll4 is necessary for arterial homeostasis after birth is unknown. Dll4 is expressed by endothelial tip cells and controls sprouting angiogenesis by activating Notch1 in adjacent stalk cells, in this context inhibiting endothelial sprouting [70]. Dll4 is strongly expressed in tumours and blockade of Dll4 in experimental tumour models leads to ineffective and uncoordinated vascular sprouting resulting in impaired tumour perfusion [74,76,77].

Jagged1

In Jagged1$^{-/-}$ mice vasculogenesis is normal. However, the remodeling of the primary vascular plexus is severely impaired leading to intrauterine death at E10 [130]. The observed phenotype is not as severe as in Dll4 or Notch1 mutant embryos. Endothelial cell specific deletion of Jagged1 recapitulates the phenotype of global deletion and results in loss of arterial differentation of SMC, indicating a direct role of Jagged1 for SMC maturation [104,105]. Morphological abnormalities in Jagged1 mutant mice appear slightly later than in Dll4 or Notch1 mutant mice, indicating that this Notch ligand becomes important when initial vascular formation has started. Jagged1 promotes endothelial sprouting and opposes the actions of Dll4 in this context (Fig. **5**) [78]. Mutations in the *Jagged1* gene are associated with Alagille syndrome [131], an inherited human disease (*see below*).

Jagged2

Jagged 2 is expressed in the arterial endothelium and upregulated after experimental balloon injury [129,132]. Analysis of Jagged2 mutant mice suggests that Jagged2 plays an essential role during limb, craniofacial, and thymic development [133]. Whether or not there is a role of Jagged2 for the vascular system remains unknown (Fig. **5**).

Adult vascular homeostasis

As described above Notch signaling critically controls arterial development and is essential for postnatal SMC physiology. It is therefore tempting to speculate that Notch is a general regulator of vascular homeostasis after birth. Hence, genes responsible for arterial identity might not only be marker genes but be functionally essential to sustain arterial environment and to prevent arterial disease.

The postnatal ablation of RBPjκ in mice leads to spontaneous and disorganized angiogenesis [134]. Notch target genes are strongly downregulated, while VEGFR2 and proliferation rates are increased in Notch-signaling deficient mice. These findings suggest that Notch suppresses proliferation to maintain vascular homeostasis. However, the Cre deleter used in this study is rather unselective [135]. Since Notch acts highly context-dependent and most Notch mutants die in utero, inducible and specific gene targeting after birth is necessary to investigate the role of Notch signaling for the postnatal vascular system. The availability of specific inducible Cre deleter lines allows the selective ablation of Notch pathway members in EC and SMC on demand. The combination of such genetic approaches with suitable animal models of vascular adaptation and growth will clarify the role of Notch pathway members for adult vascular homeostasis.

Other human cardiovascular disorders associated with defective Notch signaling

The fundamental relevance of Notch signaling for vascular physiology is apparent through inherited (cardio-)vascular diseases caused by mutations in Notch pathway genes. Besides CADASIL, which was described above, two more diseases with cardiovascular abnormalities are linked to the Notch signaling pathway.

ALAGILLE

Alagille syndrome is a human disease with an autosomal dominant inheritance. It is characterized by abnormalities in heart, skeleton, liver, eye, kidney and some other organs [136]. In most cases of Alagille syndrome mutations in the *Jagged1* gene are observed [131], however rarely mutations in the Notch2 gene were described [137]. Most *Jagged1* mutations lead to null alleles, suggesting that *Jagged1* haploinsufficiency is causative for the disease. However, data from mouse models demonstrates that Jagged1 mutations are not likely a sufficient explanation tor Alagille pathogenesis. Jagged1$^{-/-}$ mice die during embryogenesis. Jagged1 heterozygous mice develop eye defects similar to Alagille patients but lack other

abnormalities typical for the disease [130]. This indicates that Jagged1 deficiency alone can not explain Alagille disease.

However, a combined mutant mouse model heterozygous for the Jagged1 null allele and a hypomorphic Notch2 allele reproduces the gross Alagille phenotype: These mice show nearly all developmental defects characteristic for the Alagille syndrome, e.g. growth retardation and developmental abnormalities in eye, heart, kidney and the biliary system [138]. Cardiac defects in Alagille patients comprise hypoplasia or stenosis of the pulmonary artery or the pulmonic valve or tetralogy of Fallot. Since Notch signaling has been shown to be required for the differentiation of cardiac neural crest cells into smooth muscle cells, it is likely that Notch signaling defects are directly involved in the cardiac defects in Allagille patients [95]. Taken together, pathogenesis of Alagille syndrome is complex and not yet fully understood but definitely involves a deficiency in Jagged1 mediated Notch signaling.

Bicuspid aortic valve

Bicuspid aortic valves occur in 1-2% of the population and are the most common congenital heart defect [139]. Bicuspid aortic valves predispose to aortic stenosis in adulthood. Aortic valve calcification is linked to RUNX2 which is regulated by Notch signaling [140]. Heterozygous Notch1 mutations were described in two independent families with autosomal dominant bicuspid aortic valves [140], and in 4% of patients with a sporadic bicuspid aortic valve Notch1 mutations can be found [141]. Even if bicuspid aortic valve therefore does not seem to be a monogenic disease, there is strong evidence that the malformation reflects defective epithelial-to-mesenchymal transformation associated with impaired Notch1 signaling [142-144].

Conclusion

The Notch signaling pathway is essential for arterial development and growth, both during development and after birth. Many different genetic approaches demonstrate the essential role of Notch ligands, receptors and target genes for arterial identity and remodeling. However, new genetic tools enable us to specify this role to distinct Notch pathway members in specific cellular environments. More selective gene targeting design is now required to specifically adress temporo-spatial contexts of arterial growth. This will not only elucidate the molecular and cellular mechanisms how Notch controls arterial development and growth but also identify specific roles of every ligand and receptor for distinct aspects of arterial physiology. Hopefully soon this will directly translate into new therapeutic approaches for cardiovascular disease.

Table 1: Arterial and Venous Markers

Arterial ECs	**Reference**	**Venous ECs**	**Reference**
Ephrin-B2	[6, 33, 34, 145]	EphB4	[6, 33, 34]
Dll1	[43, 113]	Neuropilin-2	[146, 147]
Dll4	[113, 129]	COUP-TFII	[59, 113]
Jagged1	[129]	Msr/Apj	[148]
Jagged2	[129]		
Notch1	[129]		
Notch4	[129]		
Bmx	[149, 150]		
Neuropilin-1	[60, 146]		
Connexin 37 (Gja4), Connexin 40 (Gja5)	[60, 113, 151, 152]		
Alk-1	[153]		
Arterial SMC		**Venous SMC**	
Notch3	[98]		
Smoothelin	[101]		
Sm22a	[102]		
Ephrin-B2	[145]		

Table 2: Known Regulators of Notch Ligands in the Vascular System

Regulator	Ligand	Cell type	Setting	Reference
VEGF, bFGF	Dll1	Arterial EC	Postnatal arteriogenesis	[43]
Foxc1, Foxc2	Dll4	Arterial EC	Embryonic arteriogenesis	[52, 53]
VEGF-A	Dll4	Arterial EC Arterial EC	Arterial identity Sprouting Angiogenesis	[45, 52] [71, 73]
TNFa	Dll4, Jagged1	Arterial EC	Sprouting angiogenesis	[78]
Jagged1	Jagged1	Arterial SMC	SMC maturation	[104]
Hypoxia	Dll4			[154]

Table 3: Expression of Notch Pathway Members in the Vasculature

Gene	Expressed in	Reference
Dll1	Endothelium of middle and large arteries	[43, 123, 129]
Dll3	Not known to be expressed in the vascular system	
Dll4	Endothelium of small arteries, tumour vessels, tip cells of vascular sprouts. Absence in veins. Absence in large arteries.	[38, 46, 47, 128, 129]
Jagged1	Arterial endothelial and arterial smooth muscle cells	[95, 104, 129, 155]
Jagged2	Arterial EC	[129, 156]
Notch1	Arterial EC	[129, 157, 158]
Notch2	Not characterized yet	
Notch3	Arterial smooth muscle cells	[98, 104]
Notch4	Vascular EC	[129, 159, 160]

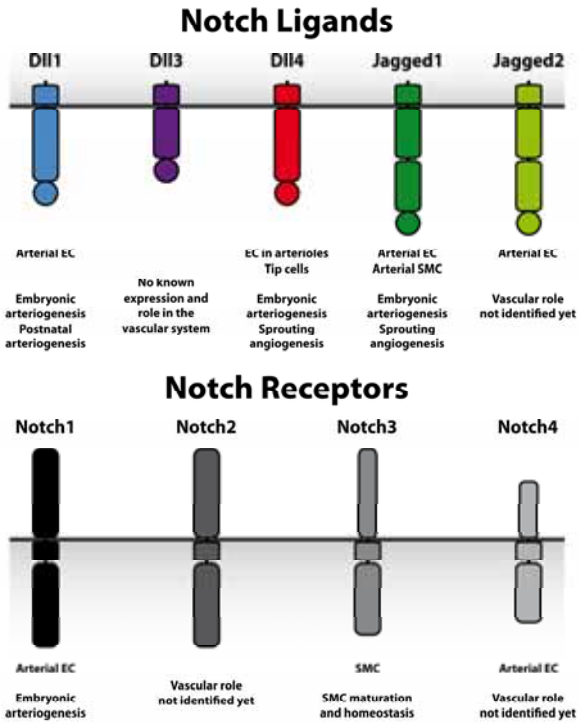

Figure 5: Comparison of Notch Ligands and Receptors. The vascular expression profile and the main functions in the vascular system are shown.

Table 4: Vascular Phenotype of General and Conditional Notch Pathway Mutant Mice

Gene	Genotype	Phenotype	Viability	Reference
Dll1	$Dll1^{+/lacZ}$ (resulting in embryos heterozygous for Dll1)	Defective postnatal arteriogenesis.	Viable and fertile	[43, 124, 125]
	$Dll1^{lacZ/lacZ}$ (embryos lack both Dll1 alleles)	Defective somitogenesis. Generalized hemorrhage from E10.5 onward.	Intrauterine death at E11.5	[125, 161]
	$Dll1^{lacZ/Dll1kineo}$ (a combination of a null allele and a hypomorphic allele)	Normal formation of myotubes, but from E13 onward severe muscle hypotrophy Normal arterial wall structure but smaller lumen of large arteries. Increased capillary branching in the skin at E17.5	Death at birth	[124, 162]
	VE-Cadherin-CreERT2 x $Dll1^{f/f}$ (postnatal knockout of endothelial Dll1)	Loss of arterial identity in EC. Normal arterial wall structure but smaller lumen of large arteries.	Long term survival was not studied	[124]
Dll3	$Dll3^{-/-}$[2]	Disorganised vertebrocostal skeleton, delayed somitogenesis. No observed vascular defects.	Death between birth and postnatal day 10	[126]
Dll4	$Dll4^{+/lacZ}$ (resulting in embryos lacking one Dll4 allele)	Severity of phenotype varies. Reduced vitelline circulation, decreased yolk sac branching, variable degree of calibre reduction of the dorsal aorta. In cases of stronger constriction also calibre reduction of the posterior cardinal vein. Some animals show enlarged pericardial space. In adult mice capillary sprouting is increased. (Phenotype is background-dependent)	A proportion of embryos dies in utero. Surviving animals are viable and fertile	[46, 71-75]
	$Dll4^{lacZ/lacZ}$ (resulting in embryos lacking both Dll4 alleles)	Normal vasculogenesis, but later on severe vascular defects in the yolk sac and embryo proper due to missing maturation of the primary vascular plexus. Disorganized and fused vessels. Severe aortic constriction. Loss of arterial and gain of venous marker expression in the aorta. Strongly resembles the $Notch1^{-/-}/Notch4^{-/-}$ phenotype.	Intrauterine death between E9.5 and 10.5	[40, 46, 47, 163]
Jagged1	$Jagged1^{-/-}$	Normal initial vascular development, but later on vascular defects due to defective plexus maturation. Impaired embryonic hematopoiesis.	Intrauterine death between E10.5 and E11.5	[130, 164]
	$Jagged1^{+/-}$	Eye defects (coloboma), no observed vascular abnormalities	Viable and fertile	[130]
	Tie2Cre x $Jag1^{f/f}$	Recapitulates the $Jagged1^{-/-}$ phenotype	Intrauterine death between E10.5 and E11.5	[105]
Jagged2	$Jagged2^{-/-}$	Syndactylia, defects in craniofacial morphogenesis and thymic development. No observed vascular abnormalities	Perinatal death	[133, 165]
Notch1	$Notch1^{-/-}$	Defects in somitogenesis. Normal	Intrauterine death	[38, 166-168]

[2] Spondylocostal dysostosis is an inheritable human disease characterized by similar vertebrocostal defects. Dll3 mutations are supposed to be causative for the disease.

		vasculogenesis, but severe vascular defects in the yolk sac and embryo proper due to defective plexus maturation. Enlarged pericardial sac, aortic constriction.	around E10.5	
	Tie2Cre x Notch1$^{f/f}$	Profound vascular defects recapitulating the general Notch1$^{-/-}$ phenotype	Intrauterine death at E10.5	[39]
	Tie2Cre x Notch1$^{+/f}$	Impaired postnatal angio-/arteriogenesis	Viable and fertile	[39, 114]
	Tie2-Cre x N1ICD^{tg} (resulting in panendothelial overexpression of the intracellular domain of Notch1)	Defective vascular remodeling, increased diameter of the aorta. AV-malformations, which are morphologically different from Notch1 loss-of-function models	Not studied yet	[169]
Notch2	Notch2$^{del1/del1}$ (targeted mutation resulting in a hypomorphic allele)	Myocardial hypoplasia, edema, disturbed hyaloid vasculature in the eye, impaired glomerular development in the kidney	Perinatal death	[136]
	Notch2$^{lacZ/lacZ}$ (embryos lack both Notch2 alleles)	Premature and increased apoptosis especially in neural tissue	Intrauterine death at E10.5	[170]
	Notch2$^{+/lacZ}$ (resulting in Notch2-heterozygous embryos)	No abnormalities reported	Viable and fertile	[170]
Notch3	Notch3$^{-/-}$	Defective maturation and arterial identity of vascular SMC. Enlarged arteries with thinner SMC layer	Viable and fertile	[99, 100]
Notch4	Notch4$^{-/-}$	No obvious phenotype under normal conditions	Viable and fertile	[38]
	VEGF-R2^{Notch4tg} (transgenic Notch4-overexpression in embryonic EC)	Disorganized vascular network, dilated blood vessels, AV-malformations	Intrauterine death at E9.5-10.5	[82, 85]
	Tie2-tTA^{Notch4tg} (transgenic Notch4-overexpression in postnatal EC)	Profound vessel enlargement and AV-shunts, ectopic arterial marker expression	Death within weeks after onset of transgenic expression	[85]
Notch1/ Notch4	Notch1$^{-/-}$/Notch4$^{-/-}$	Similar to Notch1$^{-/-}$. 50% of Notch1$^{-/-}$/Notch4$^{-/-}$embryos show a more severe phenotype than Notch1$^{-/-}$ embryos.[3]	Intrauterine death between E9.5 and E10.5	[38]
RBPjκ	RBPjκ$^{-/-}$	Defective neurogenesis, somitogenesis and vascular remodeling. Phenotype is more severe than in Dll4 or Notch1 mutants, and resembles the Notch1$^{-/-}$/Notch4$^{-/-}$ phenotype	Intrauterine death at E9.5	[40, 171]
	Tie2-Cre x RBPjκ$^{f/f}$ (resulting in EC specific ablation of RBPjκ)	Complete lack of vascular remodeling. Phenotype similar to that of Notch1$^{-/-}$ mutant mice.	Intrauterine death at E9.5	[40]
	Mx-Cre x RBPjκ$^{f/f}$	Spontaneous angiogenesis in severeal organs, increased endothelial proliferation	Postnatal gene targeting approach; it is unknown whether survival is affected	[134]
Hey	Hey1$^{-/-}$	No obvious phenotype	Viable and fertile	[49, 172]
	Hey2$^{-/-}$	Congenital heart defects	Death in the first week after birth	[173-176]
	Hey1$^{-/-}$/Hey2$^{-/-}$	Normal initial vasculogenesis, but loss of all further vascular remodeling. Enlarged pericardial sac, massive hemorrhage.	Intrauterine death at E9.5	[48, 49]

[3] The incomplete penetrance may be due to the fact that these mice were bred on a mixed background.

	$HeyL^{lacZ/lacZ}$	No obvious phenotype	Viable and fertile	[142]
	$Hey1^{-/-}$/ $HeyL^{lacZ/lacZ}$	Congenital heart defects, similar to $Hey2^{-/-}$	Death during the first postnatal days	[142]
Hes	$Hes1^{-/-}$	Severe neurogenesis defects; a role for the cardiovascular system has not been reported yet	Death between E12.5 and birth	[177]
	$Hes5^{-/-}$	no obvious phenotype	Viable and fertile	[178, 179]
	$Hes1^{-/-}$/$Hes5^{-/-}$	Similar to $Hes1^{-/-}$, but more severe. Earlier lethality.	Death around E10.5	[178, 179]
	$Hes7^{+/lacZ}$ (resulting in embryos heterozygous for Hes7)	Adult mice appear nomal, but 43% have kinked tails	Viable and fertile	[180]
	$Hes7^{lacZ/lacZ}$ (resulting in embryos lacking both Hes7 alleles)	Severe somitogenesis defects	Perinatal death from respiratory failure due to chest abnormalities	[180]

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Chapter 4. Multiple Pathways converge in the Development of a Collateral Circulation (Arteriogenesis)

Wolfgang Schaper

Abstract: Collateral vessels grow in response to arterial narrowing and occlusion. Under favorable conditions collaterals can replace part of the function of the occluded artery (40% in the canine heart, more in the peripheral circulation of some mouse strains, but some degree of muscular atrophy persists). The molecular pathways are two-pronged: inflammatory with participation of factors of the innate immune system, and shear stress dependent, which latter is the initiating factor. Blockade of inflammatory reactions leads to partial reductions of arteriogenesis. Only the inhibition of all NO production completely blocks collateral growth. Special NO donors are stimulants. A specific vascular smooth muscle mitogen induced by shear stress activated endothelium remains unknown. This holds true also for possible monocyte generated mitogens.

Introduction

The basics. Collateral vessels develop as a response to arterial occlusion. They are already universally present before occlusion as part of the arteriolar network feeding capillaries of heart, brain, intestines, kidneys and skeletal muscle [1-2]. As such they are constantly perfused and assume their role as "collaterals" only after arterial occlusion. Number and size of collateral vessels are genetically determined by the varying degree of pruning of the network of which they are a part during embryonal development [3], but genetic proof exists only for several mouse strains. The hearts of domestic pigs, rabbits, sheep and ponies exhibit collateral connections only at the capillary level [4]. Human hearts are endowed with anastomoses on all levels from arterioles to capillaries but number and size exhibit a bell-shape distribution with a 30micrometer diameter in the top class [5-7].

The shortest connections between pre- and post-occlusive branches of the arteriolar network (when present) enlarge by active growth, which is most impressive in the canine heart where the radius of grown mature sub-epicardially located vessels increases 10fold and in some cases more [1-2,8-9]. Human hearts exhibit only occasionally large epicardial collaterals. Most connections are located in the sub-endocardium and consist of large thin-walled micro-vessels forming a network. We have investigated the collateral circulation of pigs following gradual occlusion of the left circumflex coronary artery and found that although connections existed only at the capillary levels, infarcts could be prevented provided the time to occlusion was longer than necessary for the canine heart [10]. In the latter growth-enlarged collaterals carry blood into the distal part of the occluded artery, which serves, like in the normal state, as a distribution system for the blood flow to the tissues. The pressure in the distal stump of an occluded canine coronary artery is an indicator of the resistance of the collateral system provided the resistances of the micro-vascular system are equal [9]. That situation is reached by inducing maximal vasodilatation, which "clamps" the peripheral resistance to its lowest possible level. The higher the post-occlusive pressure (or peripheral coronary pressure, PCP) the lower the collateral resistance. Conversely, acutely increasing collateral flow lowers PCP, which is highest at no flow.

In the porcine heart collateral flow is carried directly into the tissue by enlarged capillaries, and it cannot use the distal post-occlusive arterial distribution system. Pressure measurements at that point tend to be very low and the diagnostic significance remain questionable.

Peripheral coronary pressure measurements in human patients have become routine in places with an interest in the coronary collateral circulation [11-13]. Pressures measured in an atherosclerotic occluded vessel that was opened by balloon angioplasty and then re-closed again with a balloon after placing a micro-manometer into the distal part of the artery, tend also to be very low, like in porcine hearts. In contrast to canine hearts, where the peripheral coronary pressure falls with increasing flow over the collateral vessels, it may rise occasionally in human patients after the vasodilatation of exercise or after chemical

vasodilators. This can be explained by assuming a rise of the arteriolar pressure in the adjacent normal myocardium, as it is typical for arteriolar pressure at vasodilatation and which is transmitted via collaterals to the point of pressure measurement. Groups of patients so tested exhibit a wide data scatter which is perhaps not only due to the degree of collateralization but also of the types of collateral, i.e., whether it had issued from pre-existent arterioles or from capillaries.

Role of ischemia. Arterial occlusion leads to tissue ischemia but growing collaterals lie usually outside the ischemic region and cannot themselves become ischemic because they carry oxygenated arterial blood [5-6,14-15]. The nucleotide spectrum of black mice after femoral occlusion, obtained by magnetic resonance, remains normal when focused at the region where collaterals grow from pre-existing precursors [16]. Aortic occlusions in the in zebra fish *gridlock* mutation are salvaged by collaterals in a fully oxygenated medium [17].

Physical forces

The initial trigger for growth by mitosis is fluid shear stress (FSS), which is caused by the increased blood flow velocity within the interconnecting network, a consequence of the large difference between the upstream and the low downstream pressure, relative to the point of occlusion[1,18]. Stretch experiments in vitro showed that circumferential wall stress rather than fluid shear stress maybe the molding force acting via the transcription factor AP-1 [19]. However, AP-1 is also activated by fluid shear stress and whereas pressure derived forces act on both the endothelium as well as on the media, fluid shear stress acts exclusively on the endothelium, which is known to play a leading role in arteriogenesis. Increased FSS leads to endothelial activation seen first as cell swelling, caused by loss of osmotic control brought about by activity changes of ion channels (Cl^-, K^+, Ca^{++}) [3,20-21]. The discussion about the role of FSS and the other stresses oscillates back and forth since about 100 years but the stronger evidence is clearly in favor of FSS since Guzman and our group could show that the high flows and low pressures observed in arterio-venous shunts resulting in increased arteriogenesis can only be explained by increased FSS [22] (see chapter 5).

Endothelial cells change phenotype into a synthetic and proliferative one with synthesis of cytokines (MCP-1) [23] and adhesion molecules (ICAM-1, VICAM-1) [1]. Adhesion and invasion of monocytes follow and endothelial mitoses become visible at day 2 [9]. FSS, only a weak force compared to pressure-related forces acting on the vascular wall, is able to activate the IIS as we have recently shown [24-25].

The cell cycle time of smooth muscle cells from the initial stimulus to completion of mitosis is about 22 hours [8]. Interventions aimed at stimulation of collateral growth must consider these constraints. Since myocardium dies within about one hour after an acute coronary occlusion, growth factor therapy arrives too late even if applied at the moment of occlusion. However, slowly progressing experimental coronary stenosis is tolerated without infarction when the time to occlusion does not fall below 3 - 6 days depending on species [26]. Un-stimulated skeletal muscle has a longer ischemic tolerance and acute femoral occlusion is tolerated without tissue damage in some species of mammals like rabbits, rats and certain mouse strains.

The **smooth muscle cells** of the media change from a contractile to a synthetic phenotype by altering expression of SM-actin binding genes, like abra, thymosin-beta 4, smoothelin, destrin and cofilin and start to divide [27] (see chapter 5). Meanwhile the **invasion of monocytes** and the expression of matrix-metallo-proteinases (MMPs) had led to the digestion of matrix and of the internal elastic lamina, provisions for the radial enlargement of the vessel [2]. Most monocytes/bone marrow derived cells are recruited from the perivascular tissue and are attracted by the chemokine MCP-1 that is strongly expressed in the smooth muscle cells of pre-existent collaterals in response to a signal emanating form the fluid shear stressed endothelium. Radial enlargement is only possible by creating new extra-vascular space, a task of invading T-cells and NK-cells [28-29].

Reduction of the number of monocytes by cytostatics (5-fluoro-uracil) or by studying animals with low monocyte counts (osteopetrosis) or knock-down with nano lipid-particles containing bisphophonates inhibits- and infusion of monocytes stimulates collateral growth [20, 30].

Monocytes do not undergo a metaplastic change into endothelial or smooth muscle cells [31]. They serve as producers of cytokines, growth factors and NO [32].

The role of NO

We noticed that excessive growth of collaterals under high FSS created by an arterio-venous shunt was inhibited by L-NAME [1], which is a non-specific inhibitor that, depending on dose, inhibits all forms of NOS. We showed recently that genetic targeting of eNOS did not inhibit collateral growth [33]. It inhibited blood flow recovery to the paw skin of the affected hind limb but this was caused by vaso-spasm, which could be unmasked by test injections of NO-donors. Deep muscle blood flow, measured by Magnetic Resonance Imaging remained normal compared to wild type animals [16,34]. Only the combined inhibition (eNOS knockouts treated with the iNOS-specific L-NIL) completely blocks collateral growth [32]. Which suggests that one isoform can substitute for the other. It is known that low activity or absence of constitutive NOS induces iNOS, which must have been the case in eNOS targeted mice that showed almost normal arteriogenesis [35]. Our data would not exclude the possibility that NO from eNOS is rapidly exhausted by persisting high shear stress during the earliest stages of collateral growth. This would favor adhesion of monocytes to the activated endothelium, which, with normal eNOS activity, would not occur. Monocyte derived iNOS and increased transcription and translation of eNOS would, at later stages, boost NO production. Since early exhaustion of NO availability is imaginable we tested the effects of NO donors and found that the close-collateral intra-arterial infusion of detaNONOate is a strong stimulant of arteriogenesis [32]. This property was not shared by the classical NO donors glyceryltrinitrate and Sin-1, the latter being a significant suppressor of collateral growth probably because of its ability to produce peroxinitrite.

The role of the innate immune system

Monocytes, accumulating close to growing collaterals, stained positive for TNF alpha [23, 36], TGF-beta, FGF-2 [37] and iNOS. The changes in the tissue concentrations of the factors resp. cells are symptoms for the activation of the innate immune system (IIS) and LPS, the classical activator of the IIS, indeed over-stimulates collateral growth [23]. Genetic targeting of TNF-alpha and its receptor partially inhibits arteriogenesis [38]. These findings strongly suggest that increased fluid shear stress, the primary trigger for collateral artery growth, is also a stimulant for the IIS, which is important for regeneration via collateral vessels. This in line with our observation that Toll-like receptors, initiators of the IIS, are up-regulated in the early stages of collateral growth (Troidl, unpublished). The observation that transplanted human hearts under full immune-suppressive medication are able to grow collaterals let the role of the IIS in arteriogenesis appear in a more complex light [11].

The role of VEGF

VEGF plays an important role in angiogenesis but its role in arteriogenesis is less clear. Smooth muscle cells play a key role in arteriogenesis if only because of their tissue mass, much larger than that of ECs. Endothelial mitosis is clearly dependent on the presence of VEGF and the role of the endothelium in directing the growth of medial smooth muscle is also undisputed. However, VEGF is not a SMC mitogen and hence cannot play a direct role in SMC mitosis. VEGF mRNA is not detectable (neither by in-situ hybridization nor by micro-array) in high fluid shear stressed collateral vessels [39]. Furthermore it was never shown that VEGF is released under in vivo conditions from fluid shear stressed endothelium. VEGF protein infusions over 7 days into the peripheral collateral circulation of rabbits did not stimulate the development of collateral vessels [40]. This contradicts earlier pharmacological studies, where massive single doses, leading also to edema, had a positive effect upon restitution of lower leg blood flow in rabbits. However interesting, this does not necessarily suggest a role of VEGF in the patho-physiological process of arteriogenesis. VEGF is up-regulated by hypoxia via the transcription factor HIF. However, it is virtually impossible that the endothelium of growing collaterals becomes hypoxic because it is constantly bathed in oxygenated arterial blood. Often collateral vessels run through tissue that does not become

ischemic itself after femoral occlusion like the upper limb muscles in the C57BL/6 mouse strain that develop to a much greater extent than collaterals of the BALB/C mouse where ischemia after femoral occlusion is extending to the upper limb but results in a far inferior degree of collateralization [16]. It is likely that VEGF production may be started by shear stress or transgenically overexpressed NOS [41], although most reports emphasize the other way round, which is difficult to reconcile with the fact that any change in fluid shear stress results in an instantaneous change in the level of NO. Studies in cultured endothelial and smooth muscle cells exposed to VEGF produce NO only after about 30 minutes [42]. The relationship between VEGF and NO may be that of a positive feedback. Studies with VEGF receptor antagonists showed that arteriogenesis is even more inhibited than angiogenesis [43]. In this study the collateral blood flow was significantly more impeded than calf blood flow. This is difficult to understand because collateral flow cannot change independently from peripheral flow since they are connected in series. In order to study collateral flow proper without interference from the periphery we created a model where the distal stump of the occluded artery, the collecting point of the collateral flow, was connected to the accompanying vein [24]. In spite of the enormous growth of collaterals in this high flow model, the peripheral flow, upon closure of the shunt, remains unaltered and only the pressure gradient along the collaterals decreases thereby increasing conductance [24]. This is not surprising because it is the peripheral flow, which is a determinant of collateral flow in the absence of additional angiogenesis.

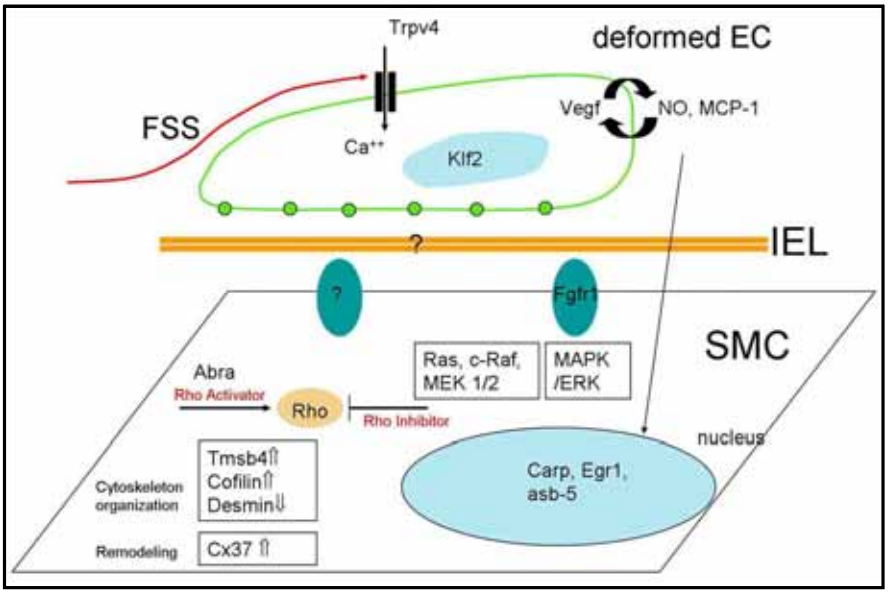

Figure 1: The figure depicts the endothelial cell deformed by fluid shear stress and secretes NO which in turn may induce VEGF (never convincingly described under in vivo conditions) leading to endothelial mitosis. The secretion of MCP-1 leads to the attraction of monocytes, which participate in the remodeling of the arteriolar structure which leads to the phenotype change of the smooth muscle of the media which eventually enter the cell cycle. Actin binding proteins play a key role. Among the transcription factors Egr-1 plays an important role, the role of CARP is still unclear. Although the smooth muscle cells exhibit an inducible FGF receptor the role of the FGF-family remains unclear because genetic targeting does not interfere with arteriogenesis. A specific SMC mitogen was so far not identified.

The quest for the elusive SMC mitogen

From many studies it has become certain that the endothelium plays a dominant role in arteriogenesis. In order to fulfill this role it has to condition the smooth muscle cells of the media to de-differentiate and to start the cell cycle and proceed to mitosis. Since direct cell-to-cell contacts between ECs and SMCs do not exist in preformed arterioles only a diffusible mitogen appears possible.

The hypothetical mitogen is produced and secreted by the endothelium, must overcome the internal elastic lamina and the extra-cellular matrix to dock on to a receptor on the SMC surface. An alternative would be that adhering monocytes provide the growth factor. Diffusible factors secreted by ECs are NO, MCP-1, endothelin 1, VEGF and, perhaps, FGF5 (FGF-1 and -2 are not secreted growth factors). Because MCP-1 is also produced and secreted by SMCs in growing collaterals, some authors believed and showed evidence for a SMC mitogenic role of MCP-1 [44]. However, the signaling chain of this chemokine does not directly lead towards canonical mitogenic processes.

A natural candidate for the SMC mitogen, necessary for structural collateral enlargement, is FGF. FGF-1 and -2 are present in heart and skeletal muscle and FGF-2 is strongly expressed in activated and adherent monocytes [37]. Infusion of FGF-2 increases growth of canine collateral vessels after coronary artery stenosis [45]. Blockade of the FGF receptor with PAS inhibits collateral growth [46]. Transgenic over-expression of FGF increases coronary blood flow and arteriolar branching [47]. However, targeted disruption of the FGF-1 and -2 genes and their double knockout does not interfere with arteriogenesis. Apparently the system is highly safeguarded and other factors substitute for the loss of FGF.

Another natural candidate is PDGF, the classical smooth muscle mitogen. However, it never showed up in our screens like differential cloning or micro-arrays with RNA from growing collaterals.

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Chapter 5. Fluid Shear Stress and its Pathways in Arteriogenesis

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Abstract: Fluid shear stress (FSS), caused by a pressure gradient between the high pre-occlusive and the very low post-occlusive regions that are interconnected by collateral vessels, is the widely accepted initial trigger of arteriogenesis. Deformation and activation of endothelial cells induces MCP-1 synthesis and leads to attraction of monocytes and T-cells. Those mononuclear cells then release proteases and growth factors, which digest the extra-cellular scaffold and provide space for the enlargement of the collateral artery. The increase in tissue mass is carried mainly by proliferation of the smooth muscle cells (SMC).

Chronically elevated Fluid Shear stress (FSS) leads to an extreme arteriogenic response resulting in a marked increase in size and number of collateral arteries. Pharmacological studies and gene expression profiling approaches were combined for the elucidation of the pathways involved in that strongly stimulated growth process, that comprises the FSS-sensing endothelium, the signal transmission from the ECs to the media and the SMC proliferation / outward remodeling.

We could demonstrate that Ca^{++} signaling plays a crucial role and represents a very early event in FSS-induced collateral growth. Further downstream, the occurrence of highly increased expression of *actin-binding Rho activating protein* (*Abra*) in growing collaterals provides link, which accounts for the remodeling during the arteriogenic process.

Introduction:

Even though several chemical or physical stimuli are discussed for arteriogenesis, the most drastic stimulation of collateral growth is achieved by an exclusive increase of FSS [1]. This was realized by adapting the established model of femoral artery ligature (FAL) in rats. We created an arterio-venous (AV)-shunt to drain most of the collateral flow into the venous system [2,3] thereby re-establishing a method invented by Holman in 1949 [4]. As a consequence, this model prevented an early growth induced decline of the FSS and resulted in a long lasting enlargement of collateral vessels. These vessels completely restored (and overshot) physiologic function of the occluded artery demonstrating that no structural restraints are the cause for an incomplete natural response (Fig**. 1**). Furthermore it allowed a detailed investigation of FSS-driven pathways and contributed to the elucidation of the mechanisms, which translate the physical stimulus into a growth response.

There are several possibilities to completely block arteriogenesis giving the first hints on the signal transduction pathways, which are involved. Inhibition of NO production as well as inhibition of the Rho pathway completely blocked the collateral growth.

Endothelial FSS-sensitive Ca^{2+} channel Trpv4 and Ca^{2+} signaling

TRPV4 – initial sensor of fluid shear stress during arteriogenesis

FSS triggered arteriogenesis leads to vast remodeling processes of the collateral network. Even though many underlying subcellular mechanisms have been identified over the last years [3,5-7], little is known about the early and initial trigger, which translates the physical force into an intracellular remodeling response. All mechanisms have in common that mechanical forces first are excerted to the inner lining of the vascular wall, the endothelium. Therefore, target genes, which are known to be mechanical stress (e.g. shear stress) sensitive, are important candidates in the context of arteriogenesis.

Tzima et al. showed that *platelet/endothelial cell adhesion molecule* (PECAM-1), *vascular endothelial cell cadherin* (VE-Cadherin) and *kinase insert domain receptor (a type III receptor tyrosine kinase)* (VEGFR2) comprise a mechanosensory complex transducing FSS into an intracellular signal [8]. Whereas PECAM-1 directly transmits mechanical force, vascular endothelial cell cadherin functions as the adaptor, which subsequently leads to activation of phosphatidylinositol-3-OH kinase via VEGFR2. Their observation was mainly based on flow-culture experiments using cultivated endothelial cells lacking VE-cadherin and PECAM-1 expression, which showed no nuclear factor kappa-B activation and a decreased inflammatory response in regions of disturbed blood flow. However, in a model where FSS is chronically elevated in the collateral network (AV-shunt) a genome wide screening of mRNA derived from collaterals showed an significant upregulation of the *transient receptor potential cation channel, subfamily V, member 4* (Trpv4) as a possible mediator of physical stimuli to intracellular signals [9].

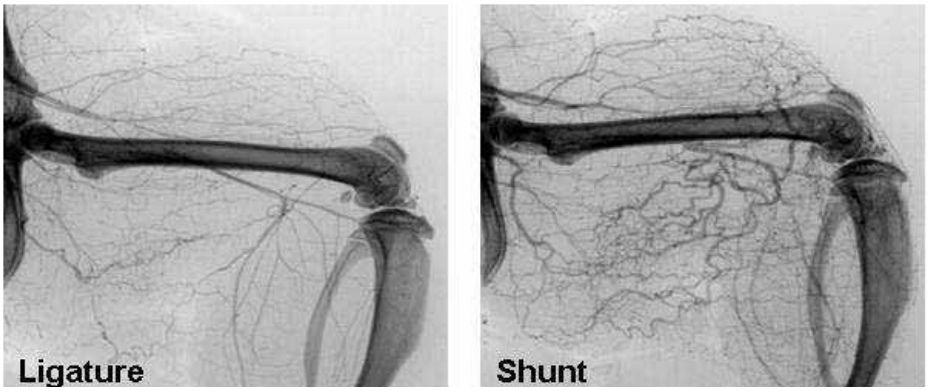

Figure 1: Angiographies of the rat hind limb 14d after the indicated treatment. Ligature depicts the natural response after femoral artery occlusion whereas after shunt treatment the size and number of the visible collateral arteries is increased. From Ref 3.

The vanilloid subfamily of Transient Receptor Potential (TRPV) cation channels have been implicated into a broad range of fundamentally physiological functions [10]. At first described by Strotmann et al. [11], Liedtke et al. [12] and Nilius et al. [13], TRPV4 is known to be a transducer of mechanical, osmotic and thermal stimuli [14-18]. In endothelial cells of the vasculature, TRPV4 is assigned an important function in regulating vasomotion [19] and, upon dysfunction, is involved in several vascular and cardiovascular pathologies [20,21].

Upon channel activation, three Ca^{2+}-dependent mechanisms are considered to play a role in transducing TRPV4-signaling across the internal elastic lamina in order to trigger vasodilatation of the smooth muscle cell layer: (1) the endothelial derived hyperpolarisation factor (EDHF) via opening of Ca^{2+}-activated K^{+}-channels, which represents an electrochemical stimulus [22]. (2) *Prostacyclin* (PGI2) synthesis by *cyclooxigenase-1*, which requires Ca^{2+}-dependent release of arachnidonic acid [15,23]. And (3) nitric oxide (NO) derived from the *Ca^{2+}/calmodulin-dependent activated endothelial nitric oxide synthase* (eNOS) [24]. In the context of arteriogenesis, it is known that eNOS-antagonsists L-NAME and L-NNA show a strong antiarteriogenic response [3,25]. Therefore, one can speculate that TRPV4-induced growth of smooth muscle cells, a crucial process during collateral formation, acts through NO because known NO-inhibitors impair arteriogenesis [3].

mRNA transcription of this Ca^{2+}-channel is constantly up-regulated in FSS-induced collaterals after AV-shunt treatment [9] (Fig. **2A** and **B**). In contrast, after FAL without AV-shunt, TRPV4 transcriptional levels are only transiently up-regulated until day 5 and drop to control levels after day 7. The chronic up-regulation (up to 14d) of TRPV4 mRNA abundance is followed by active proliferation of vascular cells, which could be demonstrated by increased numbers of Ki67 positive nuclei in the vascular wall (Fig. **2C**) and enhanced

arteriole density. In addition, administrating 4α-Phorbol-12,13-didecanoate (4αPDD) via an osmotic minipump proximal to the ligature without chronically elevated FSS (no AV-shunt) leads to increased proliferative activity of collaterals and significantly improved hemodynamics in the area of impaired blood flow (Fig. **2D** and **E**).

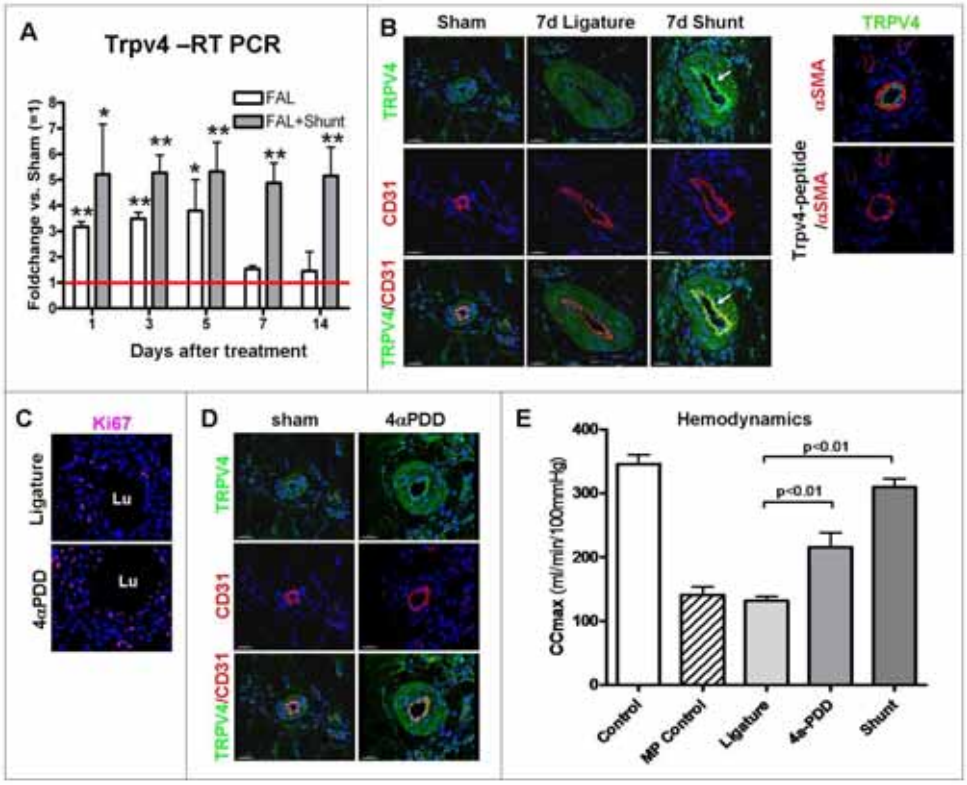

Figure 2: (A) Time course of mRNA transcription of Trpv4 in rat collaterals after ligature or shunt treatment using qRT-PCR. Relative numbers of transcripts were measured in fold changes (FC) vs. mRNA transcription of untreated control collaterals (=1; sham, red line). Note that after FAL without AV-shunt TRPV4-transcription drops to sham levels after 5d. In shunt treated rats Trpv4 in constantly up-regulated after FAL. (Values are given in mean +/- s.e.m.; n=5; *p<0.05, **p<0.01 significant vs. sham). (B) Immunofluorescence confocal microscopy of collateral vessels in rats was performed with antibodies to Trpv4 (green) and the endothelial marker CD31 (red) 7d after the indicated treatments. Double labeling demonstrated unchanged staining of the media but highly increased staining of the endothelium of shunt collaterals (arrows). In the left panel an immunoabsorption using Trpv4 peptide which was added before immunoprecipitation was performed in order to test antibody specificity. Nuclei were counterstained with Draq5 (blue). Scale bars: 40 µm. (C) Representative pictures of Ki-67 stained collateral arteries. Vessels were labeled with Ki-67 (purple). 4αPDD significantly stimulates proliferation in collateral arteries. Nuclei were counterstained with Draq5 (blue). (Lu = vessel lumen) Scale bars: 50 µm. (D) Immunofluorescence confocal microscopy of collateral vessels of rats 7d after the indicated Trpv4 modulation using antibodies against Trpv4 (green) and endothelial marker CD31 (red). 4αPDD treatment increased Trpv4 expression in the endothelium compared to sham treatment. Nuclei were counterstained with Draq5 (blue). Scale bars: 30 µm. (E) Maximum collateral conductances (CCmax) in rabbit hind limbs after indicated treatment. 4αPDD and shunt treatment significantly increased CCmax compared to minipump-solvent controls (MP control) and reaches 72.6 % of the conductance of a shunt treated rabbit. (Values are given in mean +/- s.e.m.). From Ref 9.

TRPV4 mediated calcium signaling in Arteriogenesis

It has been shown extensively that activation of TRPV4 leads to increased cytosolic calcium levels [10,12,16,17,26]. Although TRPV4 triggers vasodilatation when a fast Ca^{2+}-transient across the cell membrane leads to depolarization of the endothelium [16,23,27], TRP channels tend to have low conductances and can, therefore, also operate for a much longer time period without overwhelming the cell with Ca^{2+} [28]. Furthermore, it is known that calcium regulates many important cellular functions, including synaptic plasticity, fertilization, immune response, secretion, and proliferation [29-31]. In fact, it was recently shown that TRPV4-mediated cytosolic alterations of Ca^{2+} levels contribute to transcriptional regulation via Calcineurin (CaN) and *nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1* (NFATc1), thereby inducing osteoclast differentiation [32,33].

In line with these results NFATc1 and CaN mRNAs were strongly upregulated after TRPV4 activation in growing collateral vessels. Immunohistochemistry proved increased fluorescence signals of NFATc1 within the nuclei of growing collaterals (Fig. **3 A-C**) [34], which is known to translocate to the nucleus upon calcium-dependent dephosphorylation [35]. Our observation that NFATc1 activation depends on a constantly elevated extracellular Ca^{2+} influx via TRPV4 is supported by Rinne et al. [36], who reported that only a long lasting extracellular Ca^{2+} influx leads to NFAT activation and translocation, whereas even large amplitude Ca^{2+} signals generated by intracellular Ca^{2+} release were essentially ineffective.

Besides NFATc1, two other major and directly Ca^{2+}-dependent transcriptional regulators have been described [37]. The transcriptional repressor *Kv channel interacting protein 3, calsenilin* (KCNIP3, also known as CSEN or DREAM) translocates out of the nucleus upon Ca^{2+}-binding, thereby de-repressing its target gene promoters [38]. Overall, CSEN mRNA abundance did not change after TRPV4 activation, but immunoblotting showed that CSEN translocates from the nucleus to the cytoplasm (Fig. **3 D** and **E**) [34]. Previously, it was demonstrated that de-repression of CSEN leads to activation of *activator protein 1* (AP1) - and CREB-dependent transcription in the context of pain modulation [39]. C-JUN and FOS, which form the AP1-transcriptional complex, are up-regulated on the protein level in the nuclei of vascular and perivascular cells in growing collaterals (data not shown) [34]. Demicheva et al. demonstrated the important role of activated AP-1 during arteriogenesis [40]. Even though they postulate that cyclic stretch and not FS, which was shown by Miyagi et al. [41], triggers AP-1, they demonstrate that activated AP-1 triggers MCP1 transcription which subsequently induces the homing of monocytes, a crucial process during arteriogenesis.

In conclusion, TRPV4 activation followed by Ca^{2+}-mediated subcellular mechanisms plays a crucial role in arteriogenesis by transmitting the physical stimulus (FSS) into an active intracellular growth response thereby representing a very early event in vascular remodeling. In endothelial cells, a link between increased cytosolic Calcium levels and Ca^{2+}-mediated transcriptional signaling can be demonstrated (Fig. **4**). The signal-transmission between endothelial and smooth muscle cells is still under debate. Cell-culture experiments showed that cytokine release does not account for the transfer mechanism [9]. Small diffusible molecules like NO, which are known to pass easily through the internal elastic lamina, are most likely the second messengers between endothelial and smooth muscle cells.

Signaling pathways in the media

Despite the fact that structural changes occur in all layers of the arterial wall, the hot spot of remodeling is the media. The bulk of tissue production (up to 50-fold increase in tissue mass) proceeds in the smooth muscle cells, which transform their phenotype from a contractile into a synthetic and proliferative one. This process is further characterized by the recapitulation of an embryonic expression pattern of intracellular contractile, cytoskeletal and extracellular matrix proteins.

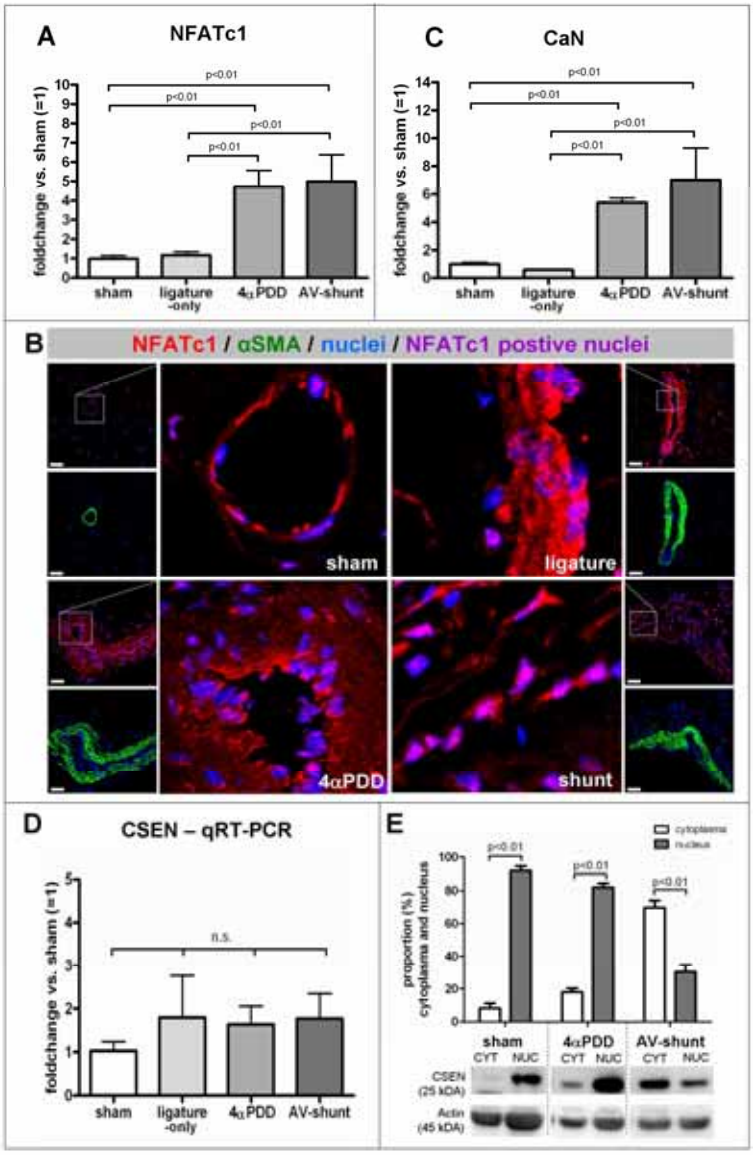

Figure 3: (A) qRT-PCR using NFATc1 specific primers. NFAT mRNA abundance was strongly upregulated after 4αPDD and AV-shunt treatment in collaterals isolated from the hind limb of pigs. (B) Immunohistochemistry using NFATc1 specific antibodies (red). Immunohistochemistry shows decreased staining of NFATc1 (red) in the cytoplasm and increased fluorescence signals of NFATc1 in nuclei (purple) of vascular cells after 4αPDD and AV-shunt treatment. (Scale bar, 30 μm). (C) qRT-PCR using calcineurin (CaN) specific antibodies. The mRNA abundance of CaN, an upstream modulator of NFATc1, was also significantly upregulated after 4αPDD and AV-shunt treatment. (D) qRT-PCR using CSEN-specific primers. No significant change in mRNA abundance after treatment with ligature-only, 4αPDD, or AV-shunt was observed. (E) Western blot analysis of cytoplasmic and nucleic protein lysates using CSEN specific antibodies. The nucleic protein fraction showed increased amounts of CSEN protein after 4αPDD treatment, which was even stronger after AV-shunt surgery. From Ref 34.

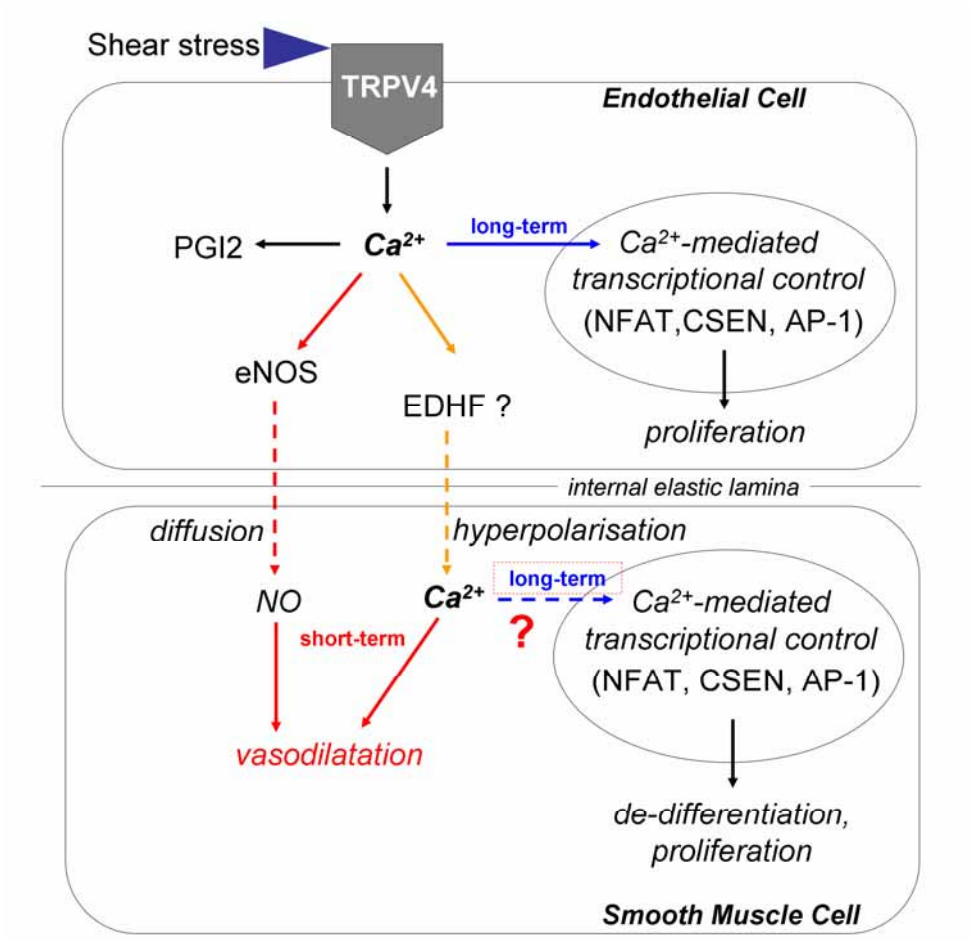

Figure 4: Schematic diagram of FSS induced calcium signaling during arteriogenesis. Whereas short-term increase of TRPV4-induced cytosolic Ca^{2+}-levels leads to vasodilatation, an increase over a longer period of time triggers calcium-dependent signaling via CaN, NFAT, CSEN, AP-1 and initiates remodeling and growth of the preformed collateral arterioles.

Phenotypic modulation of SMCs

The fact that vascular SMC are not terminally differentiated and retain the ability to modulate their phenotype to changing environmental cues likely evolved as a key mechanism to allow repair of vascular injury and adaptation of SMC contractile mass to match functional demands. Vascular remodeling during collateral growth requires proliferation of smooth muscle cells (SMC), a process which is on the other hand strongly associated with vascular disease states.

The application of chronically elevated FSS is obviously sufficient to determine an expression pattern leading to appropriate collateral growth in vivo. The search for flow dependent genes in endothelial cells under static or flow conditions *in vitro* will not adequately represent the complex situation. A gene expression pattern of FSS-stimulated growing collaterals, which were dissected from the surrounding muscle, is expected to reflect the unique arteriogenic response (Fig. **5A**). This profiling revealed down-regulation of genes with the highest specificity for SMCs, whereas those genes, which are expressed in all three muscle types

during development, are either unchanged or up-regulated. Chronically elevated FSS coincides with a prolongation of the dedifferentiated state of smooth muscle cells (Fig. **5B-E)**. Differentiation markers *smoothelin* (Smtn, 0.4-fold) and *smooth muscle myosin* (Myh11, 0.5-fold) are significantly downregulated in FSS-stimulated growing collaterals. In contrast, a re-expression of cardiac, fetal or skeletal isoforms of proteins was observed (*Acta1* 12.2-fold; *Actc1* 6.1-fold.; *Tpm1* 12.0-fold; *Tuba*1 2.4-fold; *Myh6*/*7* 9.4-fold; *Desmin* 4.6-fold).

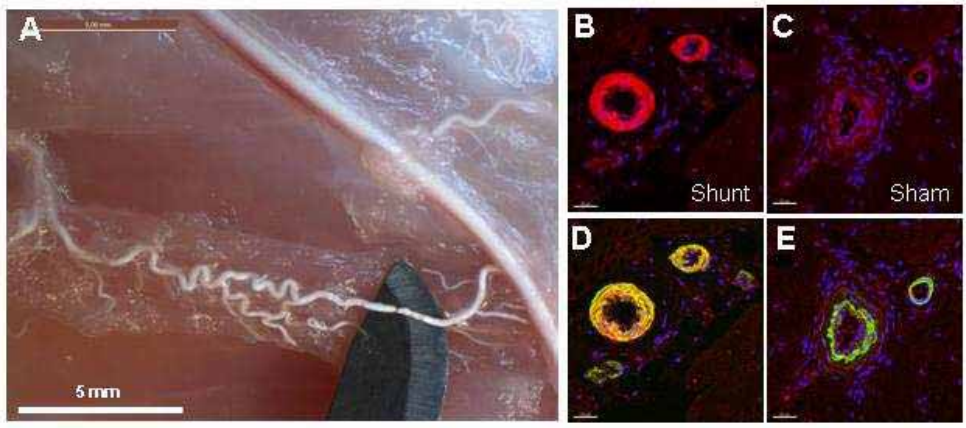

Figure 5: A: Superficial adductor muscle collateral arteries connecting the A. saphena with the A. femoralis visualized under the stereo-microscope. Midzones of these collaterals were dissected from the surrounding muscle and subjected to RNA isolation. B-E: Immmunostaining showing the de-differentiated state of FSS-stimulated collaterals. red: Smoothelin, green: α-smooth muscle actin, blue: nuclei. D+E merge

The transcriptional control of these events is still under investigation. Differentiated SMC are defined by an array of contractile and cytoskeletal proteins as well as transcription factors and signal transducers. A partial transcriptome of a smooth muscle cells is reviewed in [42]. A large part of SMC restricted genes contain one or more CArG elements representing binding sites for serum response factor (SRF). Even though SRF is expressed in all cell lineages it only activates transcription of SMC-restricted contractile genes in SMC by recruiting the potent transcriptional co-activator myocardin, which is exclusively expressed in SMC and cardiomyocytes. The paradoxon of SRF's ability to simultaneously activate transcription of genes involved in opposing cellular processes such as differentiation and proliferation reflects the complex network of molecular pathways underlying arteriogenesis.

The transcription factor early growth response 1 (Egr-1), which we found upregulated during collateral artery growth [43], participates in this ensemble of signal transduction [44]. Five serum response elements (SRE) with adjacent binding sites for the transcriptional co-enhancer Elk-1 (nuclear target of the Raf/MEK/ERK cascade) have been identified on the Egr-1 promoter [45]. It has further been shown that SRF is involved in the transcriptional regulation of Egr-1 [46] and that FSS induces the mRNA of Egr-1 in endothelial cells (EC) *in vitro via* the Raf/MEK/ERK pathway [47]. Egr-1 is a key regulator in growth processes [48] interconnecting upstream growth factor (GF) signals in ECs [49] with Egr-1 mediated upregulation of downstream GFs, for instance in response to FSS [50]. Furthermore, Egr-1 controls cell cycle progression *via* cyclin D1/E or cell division cycle 20 gene (cdc20), all of them being among the identified Egr-1 *in vitro* downstream genes [51,52]. SMC proliferation and phenotype switch, the two essential processes in arteriogenesis, are strongly dependent on downregulation of the transcriptional repressor splicing factor-1 (SF-1) [53] and SMC proliferation requires the presence and accurate function of Egr-1 [54]. In this regard, recent *in vitro* data indicate that Egr-1 is responsible for this downregulation in response to GF stimulation [53,55]. As stated above, myocardin co-enhances SRF mediated transcription of SMC-restricted contractile genes, maybe alternating with Elk-1 related gene (Egr-1)

transcription. Both co-enhancers translocate into the nucleus depending on the signal transduction cascade being activated upstream and the process involved. So it appears that it is not SRF but rather the co-enhancers myocardin and Elk-1 recruiting and competing for SRF within the nucleus.

Irrespective from the transcriptional control, the cytoskeletal integrity as well as the actin polymerization status itself could also account for structural changes. Changes in actin dynamics are hallmarks for arteriogenesis: The phenotypic transition in smooth muscle cells during arteriogenesis from the contractile to the synthetic and proliferative state is characterized by the lack of actin filaments, which is a result of down regulation of actin transcription as well as the degree of actin polymerization. In our experiments of chronically elevated FSS we prolonged the dedifferentiated state of smooth muscle cells that is characterized by differential expression of the actin-(de)polymerizing proteins (destrin, cofilin1, cofilin2, and transgelin2) [2]. A specific staining of F-actin and G-actin supports this observation: F-actin is more fragmented whereas G-actin accumulates in shunt vessels compared to sham treated collaterals [56]. Disruption of actin cytoskeleton mediates the loss of tensile stress induced early phenotypic modulation of VSMC in organ culture [57] and this is accompanied by accelerated actin cytoskeleton dynamics and down-regulation of myocardin and SRF. Tightly coupled with the changes of actin polymerization is the Rho signaling pathway. We knew from previous studies that the inhibition of Rho-kinase by Fasudil completely blocked the effect of FSS on collateral growth [3].

A co-transcriptional factor, which exhibits strong up-regulation in rabbit and mice was the cardiac ankyrin repeat protein carp (ANKRD1) [58]. Apart from its ability to regulate growth factor expression during embryonic development its role during arteriogenesis in the adult organism remains unclear. Targeted deletion of ANKRD1 did not show a delay in blood flow recovery after femoral artery occlusion and adenoviral over-expression did not improve collateral growth.

A common initiator: Abra

In a genome-wide screening of mRNA abundance in growing collaterals of rats we identified *actin-binding Rho activating protein* (*Abra*). Abra (also known as Stars) is a muscle specific actin-binding protein capable of stimulating *SRF*-dependent transcription through a mechanism involving *RhoA* and actin polymerization [59]. In cardiac tissue *Abra* mRNA is up-regulated in response to pressure overload [59,60]. Recently it was shown that Abra is involved in human skeletal muscle hypertrophy and atrophy [61]. Forced overexpression of *Abra* in mouse heart tissue results in an increased sensitivity to biomechanical stress stimuli leading to cardiac hypertrophy [62]. However, its role in blood vessels has not been determined. In light of the requirement of Rho signaling during arteriogenesis, we hypothesized that a fluid shear stress induced up-regulation of *Abra* initiates collateral remodeling. And indeed, adenoviral gene transfer of *Abra* improved arteriogenesis, thereby partially substituting for FSS. Cell culture studies demonstrated an *Abra*-triggered proliferation of smooth muscle cells through a mechanism that requires Rho signaling [56].

The well-established abilities of *Abra* to activate Rho signaling, to influence actin dynamics as well as cytoskeletal integrity, and to initiate SRF-dependent gene transcription [59] provide potential mechanisms to account for the improvement of collateral growth. We know from previous studies that the Rho-pathway is involved in flow-related remodeling of small arteries [63] and in particular in arteriogenesis [3] because the Rho kinase blocker Fasudil abolished the beneficial effect of shunt treatment in rabbits. Increased RhoA expression in FSS-stimulated collaterals as well as in Ad_Abra treated collaterals support a causal connection of Abra and RhoA in arteriogenesis. However, Abra promotes the formation of F-actin and Rho activation leads to actin polymerization. This apparent contradiction corresponds with and extends previous findings derived from forced Abra overexpression in the heart. Increased expression of Abra in response to stress stimuli – FSS in our model – may initially serve as a compensatory response to increased actin content to maintain cytoskeletal integrity and sustain arterial function [62]. But excessive expression leads to adaptation, which results in adverse cardiac remodeling in mouse models of cardiac hypertrophy [62] or, as we could demonstrate in rat and rabbit models of FAL, to beneficial collateral growth. Interestingly,

Abra over-expression in both models is accompanied by activation of SRF-dependent fetal cardiac genes [62]. Abra initiates SRF dependent transcription. Apart from previous findings of SRF-dependent transcription during arteriogenesis (reviewed in [64]), we were able to demonstrate that SRF itself is up-regulated under conditions of high FSS. RhoA-dependent regulation of the actin cytoskeleton selectively regulates SMC differentiation marker gene expression by modulating SRF dependent transcription [65].

In summary, the "Arteriogenesis" pathway, which remodels a preexistent arteriole into an artery, originates at the shear stressed endothelium and induces a multitude of morphological, biochemical and molecular changes in endothelial and smooth muscle cells. We have discovered essential pathways, which contribute to FSS-sensing as well as to smooth muscle proliferation, but the fundamental event that initiates the mitogenic stimulation and distinguishes the arteriogenic outward remodeling from the negative atherosclerotic inward remodeling has not been unraveled.

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Chapter 6. Impact of Shear level and Cardiovascular Risk Factors on Bioavailable Nitric Oxide and Outward Vascular Remodeling in Mesenteric Arteries

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Steven J. Miller

Abstract: Flow-mediated remodeling is a fundamentally important phenomenon which occurs throughout the arterial vasculature including the small mesenteric arteries. The accessibility of the mesenteric vasculature facilitates the use of intravital techniques, and hemodynamic measurements are possible as well as direct assessment of peri-arterial NO and H_2O_2 concentrations. The mesenteric arterial branching pattern provides the ability to place ligations and make graded flow alterations in specific vessels, unlike other organs with a more complex vascular network. Such studies with the mesenteric model have established that the magnitude and rate of luminal expansion, as well as cell proliferation, depend upon shear level and that outward remodeling is associated with increased MMP2 expression/activation. Animal models of chronic oxidative stress demonstrate profound suppression of flow-mediated outward remodeling that is associated with abnormal flow-related protein expression, including eNOS. Anti-oxidant therapies restore the capacity for flow-mediated remodeling and NOS inhibition prevents their beneficial effect. However, direct measurements of NO reveal that peri-arterial NO is not decreased in these models but rather increased by elevated H_2O_2. Thus, the beneficial effect of anti-oxidant therapies may result from some mechanism other than improving bioavailable NO, such as reversal of an oxidant dependent, NO-insensitive state. Additional studies are warranted to investigate the mechanisms mediating the collateral growth impairment which occurs in humans as well as animals, as the results could facilitate development of novel therapies. The unique advantages of the mesenteric model may be useful in further elucidating these mechanisms and evaluating potential therapies.

Importance of flow-mediated remodeling

Vascular remodeling refers to structural changes within the vascular wall that are not due to active smooth muscle constriction or dilation. Blood flow and wall shear stress have long been known to be important for luminal expansion of vascular structures during embryonic development and early postnatal growth. Even in mature animals where low rates of cell turnover indicate that major vascular structures are extremely stable [1-2], individual conduit and resistance arteries are known to undergo remarkable changes in luminal and wall dimensions in response to altered hemodynamic forces. Such changes in vascular structure are known to occur during menstrual cycles, pregnancy, muscle disuse and exercise training, weight gain, and in various disease states. Significant evidence exists that luminal diameters of large through small arteries and arterioles are determined by wall shear stress. Chronic alterations in shear forces are associated with structural luminal expansion or retraction appropriate to restore wall shear forces [2-4]. Flow-mediated remodeling occurs in the aorta [5], carotid [3,6-7], iliac [4], middle cerebral, basilar [8], radial [9], ulnar [10] and uterine [11] arteries and skeletal muscle arterioles [12] and thus appears to be universal throughout the arterial system. Although it is widely recognized that wall shear stress is one of the hemodynamic forces that are fundamentally important in the regulation of vascular structure and matching perfusion with metabolic need, controversies exist to this date in the specific stimuli, sensors, mediators and remodeling processes involved [13].

One area where our knowledge is specifically limited and additional investigation needed is the impact of various pathological and disease states on flow-dependent remodeling. Langille [1] has reviewed the potential role of flow-related remodeling in atherosclerosis, intimal proliferation, and post-stenotic dilatation and provided evidence that flow-mediated outward remodeling is suppressed with normal maturation and aging [14]. An additional condition where flow-dependent remodeling is considered to have major significance is the enlargement of pre-existing vessels which form collateral pathways in arterial occlusive

disease. Human and pre-clinical studies have shown that the vasculature is capable of full compensation for major arterial occlusion in the peripheral circulation through collateral growth, but also indicate a profound impairment of collateral growth in the presence of risk factors for vascular disease, especially aging (reviewed by Ziegler et al. [15]). Abnormal levels of nitric oxide (NO) and reactive oxygen species (ROS) are associated with vascular risk factors and may contribute to impaired collateral growth, but the role of these reactive molecules is controversial, even in the context of successful collateral growth. Because ROS and NO mediate many signaling events, transcription factor activation, gene expression, MMP activation, and cell growth (Fig. **1**), understanding their role in flow-mediated remodeling is fundamentally important.

The most common site of peripheral arterial occlusion is the lower extremity. Current animal models of arterial occlusion and collateral growth in the hindlimb have limitations in the ability to measure hemodynamic forces and identify primary collaterals for morphometric and biochemical analyses. Such measurements are fundamentally important in determining mechanisms responsible for flow-mediated remodeling and its impairment. In this chapter, we review studies performed in simpler models in which the arterial flow through small resistance arteries can be varied, hemodynamic measurements are possible, and isolation of the experimental and control arteries more easily performed. In addition, direct measurement of NO and ROS are possible with existing technology for intravital studies.

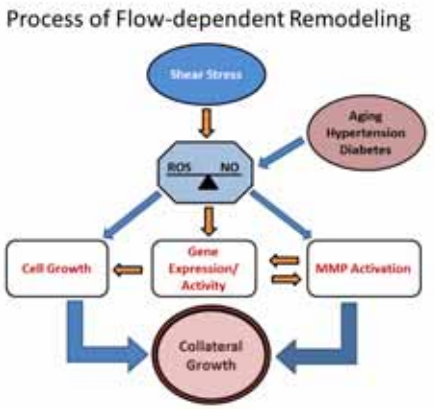

Figure 1: Chronic alterations in shear stress stimulate vascular remodeling through multiple mechanisms and pathways associated with matrix degradation and deposition; cellular hypertrophy, hyperplasia, recruitment and apoptosis; and changes in molecular expression and activation. Shear stress alters the production of ROS and NO which may influence all of these processes. Vascular disease and risk factors also influence ROS and NO levels and impact the remodeling process.

Development of the Mesenteric Model

The mesenteric model of arterial occlusion and collateral growth was originally developed after initial studies in the rat hindlimb convinced us that the questions we wanted to investigate could be addressed more directly and clearly in a different model.

Studies of the microvasculature in tissues distal to the site of arterial occlusion are controversial. While many studies report angiogenesis, this does not occur in all animal models [16] and may depend upon the specific time investigated [17]. In addition, evidence from humans with peripheral arterial occlusive disease suggests that there is a decompensation or regression of small arteries and arterioles [18-19]. Very few studies have assessed the hemodynamic significance of collateral versus microvascular adaptations. In 1994 and 1995, we reported three studies in the rat which investigated the role of collateral and distal microvascular resistances after abrupt femoral artery occlusion. We had begun with the hypothesis that microvascular adaptations would be an important component of vascular compensation to arterial occlusion, and that microvascular resistance distal to the occlusion site would be decreased by an increase in arteriolar diameters and numbers. This hypothesis was based upon the knowledge that small arteries and large arterioles normally have the major role in the regulation of vascular resistance and tissue perfusion in the peripheral circulation [20-23]. Our first reported study found that the initial compensation to abrupt femoral artery occlusion occurred in collateral vessels rather than the distal microcirculation [24]. Within seconds, both arterial inflow and distal pressure began a recovery from the nadir

which occurs immediately after abrupt arterial occlusion. The increase in distal pressure indicated a greater decrease in collateral than distal microvascular resistance. Administration of nitric oxide synthase inhibitors prevented the recovery and suggested that the compensation was largely due to NO mediated dilation of collateral arteries, presumably in response to elevated blood flow and shear stress. Subsequent studies at 1 and 8 weeks post-occlusion indicated progressive compensation as hindlimb vascular resistance decreased. This decrease occurred entirely in the collateral circuit rather than the distal microcirculation (Fig. **2**). These data are consistent with early responses to femoral occlusion reported for the canine hindlimb [25] and human lower extremity [26] and also for chronic adaptations in the canine and murine hindlimbs [27-28].

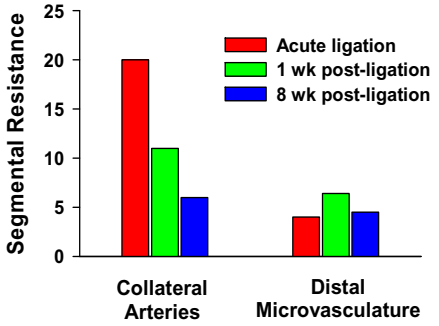

Figure 2: Resistances of the collateral circulation and distal microvasculature (mmHg*(ml/min)-1) after rat femoral artery ligation. The figure shows distal microvascular resistance remains unchanged as collateral resistance progressively decreases after femoral artery occlusion. From Ziegler et al. [15], based on our published studies [38,39].

At this point, we were interested in investigating the role of hemodynamic forces and metabolic factors in the vascular remodeling processes which resulted in rapid decreases in collateral resistance without apparent compensation in the distal microvasculature. We were unable to develop a viable experimental approach to investigate these areas in the hindlimb. Microscopic computed tomography was not available and assessing changes in microvascular diameters, lengths, and numbers would be difficult due to the complex branching patterns and three-dimensional arrangement in multiple calf muscles. Longland's work [29] had not only demonstrated the existence of multiple collateral pathways in the hindlimb but also observed that many begin to enlarge, but that subsequently most undergo regression. These hindlimb collaterals are embedded within and between muscles, so even if we could identify them, their anatomical location would prevent in vivo measurements of pressure and flow to assess hemodynamic stimuli. In addition, we would not be able to determine with certainty if a given vessel were in the process of luminal expansion or regression. These limitations led us to consider alternative models. Our previous work had demonstrated the utility of the rat terminal ileum for evaluation of adaptations in the microvascular network that occurred during juvenile growth and maturation [30-32]. The entire intestinal vasculature could be visualized from small feed arteries to small arterioles and even muscle layer capillaries. Comparisons made of exactly the same tissue regions and vessels at multiple times demonstrated a decrease in vascular density occurred during rapid post-natal growth which was due entirely to tissue expansion rather than a loss of microvessels. With this preparation, the gain and loss of individual arterioles between observations can be determined as well as changes in diameter. In addition, the small mesenteric arteries are readily accessible for in vivo measurements including pressure and flow.

Characteristics of the model and comparison to the hindlimb

Because of the advantages indicated above, we began to develop models of mesenteric artery occlusion. Our initial results indicated that arterial ligations could be made without bowel infarction and that significant adaptations were observed in both arterioles and small arteries as shown in Fig. **3** and **4**, respectively. We further developed and characterized the model in which the small ileal arteries experienced increased blood flow and underwent enlargement, as this branching order between a major artery (superior mesenteric artery) and the distal feed arteries is similar to the branching order of arteries which function as primary collaterals in the human leg [33-35] (as shown in Fig. **5** and reviewed by Ziegler et al. [15]). In addition, these vessels are more easily isolated than the microvessels embedded in tissue and provide

greater amounts of tissue for biochemical analyses. A schematic representing this model is presented in Fig. **6**. The major characteristics of this model, including hemodynamic alterations and the early adaptations in the collateral arteries which experienced elevated flow, and the distal microvasculature were reported in 1996 [36]. These results are reviewed below and also compared to the hindlimb.

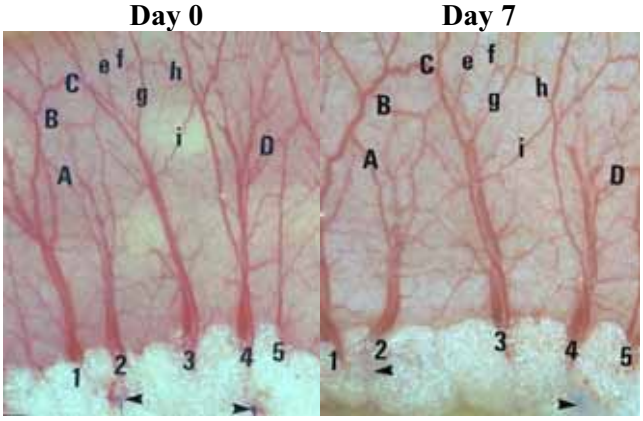

Figure 3: Arteriolar collaterals in the intestinal wall. Numbers 1-5 near the bottom of the micrographs identify 1st order arterioles arising from the marginal artery. Marginal artery ligations were made between arterioles 1 & 2 and 4 & 5 at sites indicated by arrowheads. Photomicrographs were made under maximal dilation after ligation (Day 0) and 1 week later (Day 7). Interconnecting arterioles which span the occlusion to form collaterals and would be expected to experience increased flow are identified in capital letters and are enlarged (A-D). Arcading arterioles which would not form collateral pathways are identified in lower case letters and are not enlarged.

Arterial pressure distal to the occlusion

A clinically significant arterial stenosis or occlusion in the peripheral circulation is typically manifested by a reduced arterial pressure distal to the site of occlusion or stenosis [37]. In our model of ileal artery ligation, arterial pressure at the center of the collateral-dependent region was substantially reduced to about 30 mmHg by arterial ligation and then gradually recovered [36], similar to the distal pressures measured after femoral artery occlusion in the rat hindlimb [38-39].

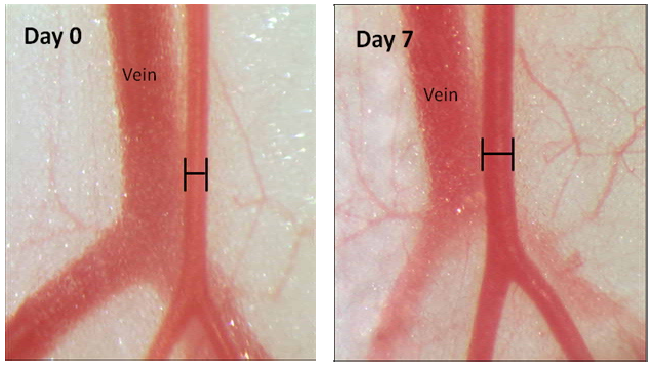

Figure 4: Small arterial collateral in mesentery. Occlusion of adjacent arteries (Fig. **6**) resulted in increased blood flow in the patent arteries which functioned as collaterals for the intestinal regions originally perfused by the occluded vessels. One such collateral ileal artery is shown initially after ligation and 7 days later. Calipers placed in the micrographs at the exact location illustrate vascular enlargement from Day 0 to 7. Both photographs were taken at the same magnification. The ability for repeated diameter measurements is a significant advantage of the mesenteric model.

Distal tissue perfusion

Tissue perfusion assessed by microspheres indicated that intestinal blood flow at the center of the collateral dependent region was similar to control regions at rest, but was significantly decreased with maximal dilation [36]. This is similar to what is observed in human claudicants and some rodent models of single vessel occlusion where flow reserve is eliminated but resting perfusion is normal [40].

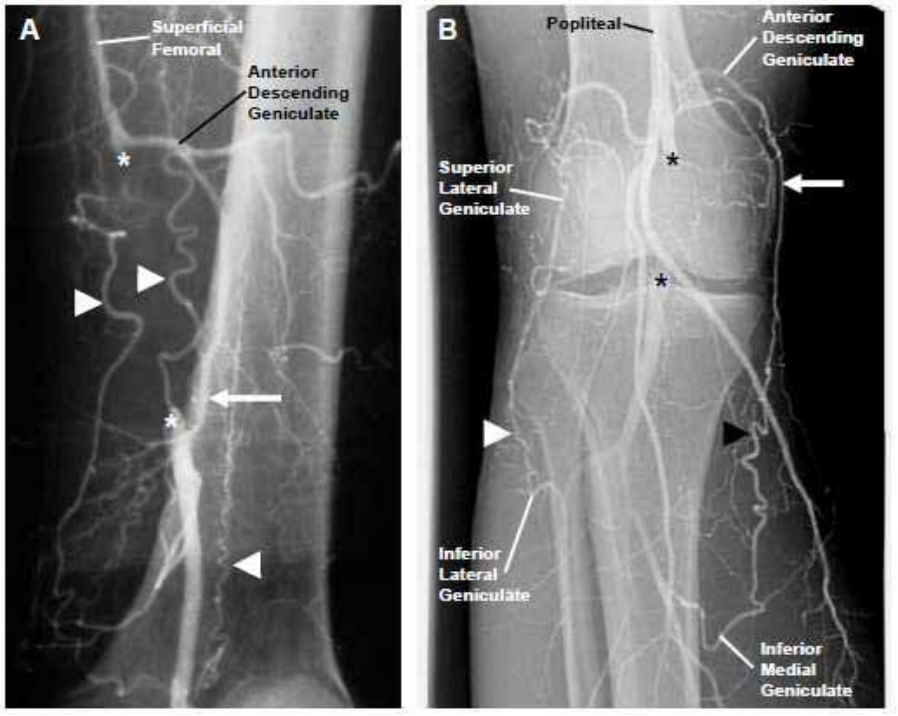

Figure 5: Lower extremity human angiograms from Ziegler et al [15] demonstrate natural collateral development subsequent to focal arterial occlusion. Collateral vessels are observed bypassing the site of a superficial femoral artery occlusion (A) and a popliteal artery occlusion (B). The occlusion is bounded by asterisks. Arrows and arrowheads identify collateral arteries with minimal and significant tortuosity, respectively. The collaterals providing distal reconstitution arise from small pre-existing geniculate arteries, consistent with published reports [33-35].

Collateral and microvascular adaptations

Distal arterial pressure increased ~30% during the first week post-occlusion [36]. This increase in distal arterial pressure is consistent with what is observed after occlusion of the femoral, carotid, and coronary arteries [41] and indicates a greater compensation in the collateral arteries than the distal microcirculation. Evaluation of the arteriolar network for arteriolar numbers and diameters at the center of the collateral dependent region (Fig. **6C**) indicated no significant changes within the first week after occlusion [36]. During this same time, there was ~30% diameter increase in the mesenteric collateral vessels which experience elevated flow. These results are consistent with the measurements of collateral and distal microvascular resistances in the rat and cat hindlimbs after femoral artery occlusion [27,38].

Relationship between shear force and wall remodeling

When we began our studies with the rat mesentery, shear stress had been shown to regulate luminal dimensions in conduit arteries and to impact wall structure in these same vessels, but there were no studies available on remodeling in small resistance arteries under known alterations in wall shear forces. For these vessels, the stimulus level necessary to initiate structural outward remodeling and the degree of luminal expansion that would result from a

given increase in shear stress were unknown. Neither was the impact of shear level on the specific nature of the remodeling process known.

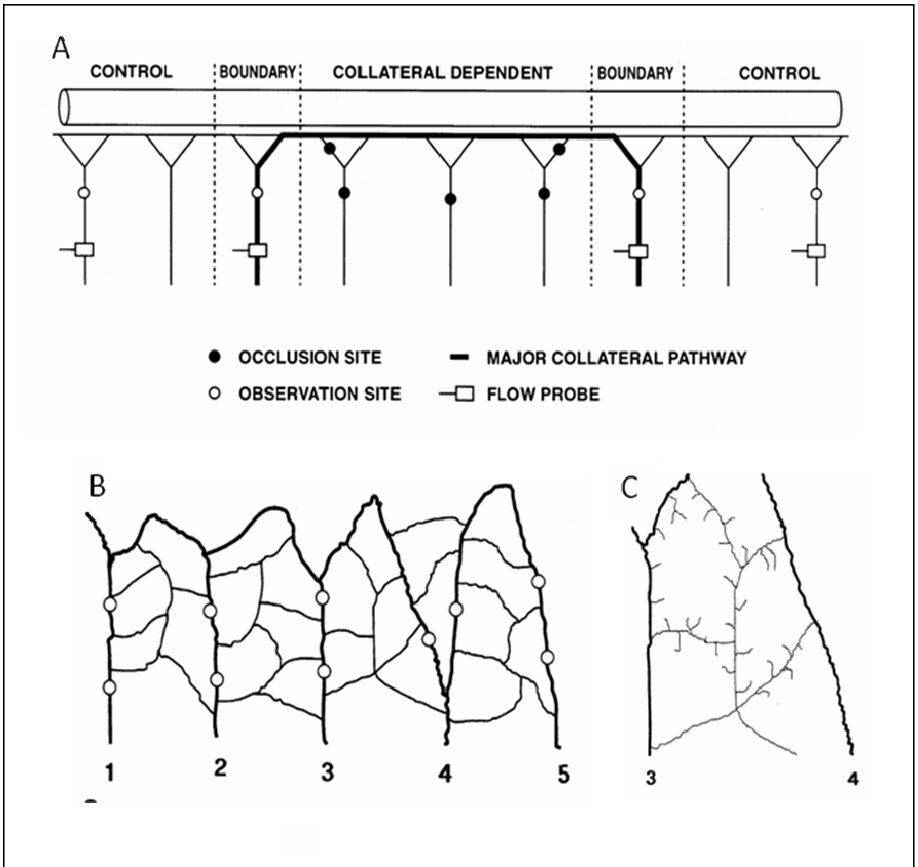

Figure 6: Mesenteric artery occlusion model. These sketches from Unthank et al.[36] represent the model used to assess collateral and microvascular adaptations. In A, the collateral pathway is shown by thick black lines and sites of occlusion, diameter, and flow measurement are indicated for collateral and control vessels. Pilot studies indicated elevated flow occurred only in the ileal arteries immediately adjacent to the ligated arteries. For each animal, micrographs and video recordings were made similar to the sketches shown in B and C to identify changes in the number of first- through third-order arterioles and arteriolar diameters under maximal dilation at both the initial and final times of observation.

We performed morphometric analysis of arterial cross-sections to obtain insight into the remodeling processes involved. Figure **7** shows representative cross-sections of control and collateral arteries from the same animal. In Figure **8**, data from our published work is summarized to show changes in luminal and medial areas of the experimental artery which experienced chronically elevated blood flow. The results demonstrate that luminal and medial cross-sectional areas relative to same animal controls are significantly increased at 1-12 weeks post-occlusion and that most of the increase occurs during the first week. This indicates that, even at the most rapid phase of flow-related luminal expansion in these small resistance vessels, the remodeling process is hypertrophic and there is an increase in wall components. Medial thickness was also increased [2] such that the relationship between

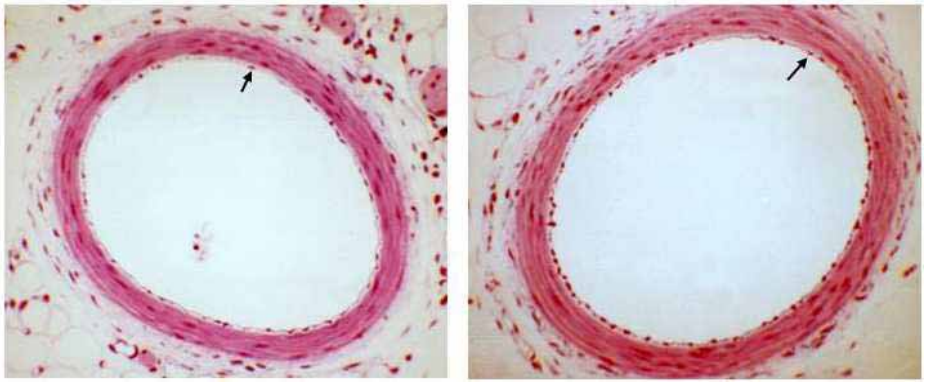

Figure 7: Representative cross-sections of control and collateral ileal arteries at one week post-ligation. Vessels were perfusion fixed at the mean systemic arterial pressure and embedded in plastic due to the superior histology and nuclear numbers and densities. The most remarkable difference between the control and collateral arteries was the increase in intimal cell nuclei (arrows identify single intimal nuclei in each image).

medial thickness and luminal radius remains unchanged. This suggests that the specific remodeling response was acting not only to restore normal wall shear forces but also to preserve circumferential wall stress, even during the early and rapid phase of luminal expansion.

The medial layer hypertrophy is similar to other reports of flow-mediated outward remodeling [6,8,42-43]. The rapid luminal expansion is consistent with the increased collateral diameter and swift improvement in perfusion observed in the rodent hindlimb [44]. It is also consistent with reports from humans indicating significant collateral growth can occur within days to weeks after either abrupt arterial ligation in wounded limbs or atherosclerotic occlusion [35, 45].

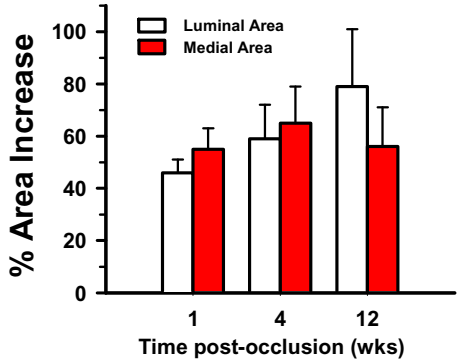

Figure 8: Percent increase in cross-sectional luminal and medial areas of collateral vessels experiencing elevated flow relative to same animal controls at 1, 4, and 12 weeks post-occlusion. Data from Unthank et al. [2, 46].

Wall constituents

To obtain insight regarding the mechanisms involved in the hypertrophic remodeling response, we determined intimal and medial cell numbers and densities by nuclear counts in arterial cross-sections [2, 46].

Endothelial cell response. The most apparent and dramatic aspect of the mesenteric collateral arterial remodeling was the response observed within the intima (Fig. **7**). Examination of arterial cross-sections, longitudinal sections, and whole vessel imaging revealed a remarkable increase in the number of intimal nuclei. The nuclei were uniform in size and shape with the longitudinal axis oriented parallel to the axis of flow, consistent with an endothelial phenotype. As illustrated in Figure **9**, after 1 wk of elevated flow there was almost a 100% increase in the intimal cell nuclei as percent of same animal control vessel. This was reduced slightly at 4 wks and significantly at 12 weeks. Intimal cell density (nuclei per 100 µm luminal perimeter) was increased 60% at 1 wk, remained elevated at 4 wks, but was reduced and similar to control vessels at 12 weeks [2,46]. Endothelial cell proliferation and

recruitment occurs in conduit arteries exposed to chronically elevated shear and precedes the luminal expansion [47-48]. As shown in Fig. **10** and **11**, this response observed in mesenteric collaterals is similar in nature to what we have observed and reported for primary collaterals in the murine and porcine hindlimbs [15,44].

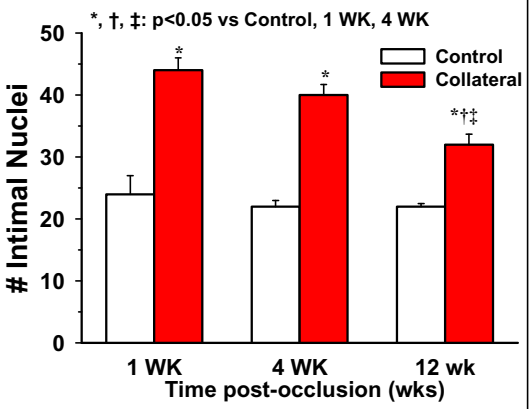

Figure 9: Number of intimal nuclei in cross-sections of control and collateral arteries at 1, 4, and 12 weeks after abrupt flow elevation by occlusion of adjacent ileal arteries. Data from Unthank et al. [2,46].

Smooth muscle cell response. Unlike the response observed in the intima, medial cell nuclear number was not significantly increased at one week (Fig. **12**) post-occlusion despite a >50% increase in medial area. Consequently, medial cell density was decreased ~30% [2]. By 4 wks however, there was an 80% increase in the number of medial nuclei in arterial cross- sections, and medial cell density was slightly increased relative to control arteries [2]. At 12weeks the absolute number of medial nuclei compared compared to controls was increased 35% and medial cell density was slightly reduced [46]. Our results demonstrating smooth muscle cell hyperplasia at 4 weeks after chronic flow elevation in mesenteric collateral arteries are consistent with studies of flow-mediated outward remodeling in a similar rat mesenteric model and in carotid and basilar arteries [8,42-43]. Our data, however, suggest that smooth muscle cell hypertrophy both precedes and follows the hyperplastic response.

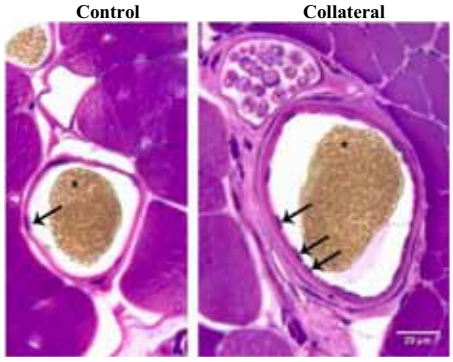

Figure 10: Representative cross sections demonstrate typical luminal expansion and increased endothelial cell numbers in mouse hindlimb collateral arteries compared with control arteries at 14 days after femoral artery ligation. Arrows identify some of the intimal nuclei. The asterisk identifies the presence of Microfil in the lumen. From Distasi et al. [44].

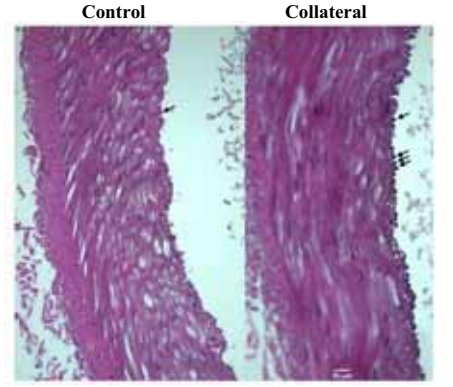

Figure 11: Arterial cross-sections 4 wks after femoral artery ligation in the porcine hindlimb. Note the remarkable collateral intimal response. All intimal cells displayed positive immunoreactivity for von Willebrand factor. Arrows identify endothelial nuclei. From Ziegler et al. [15].

Matrix metalloproteinase (MMP) expression/activity. The expression and activation of MMP-2 and MMP-9 were investigated to determine their potential role in the remodeling of small resistance arteries subjected to a degree of wall shear stress elevation that might occur

under physiological conditions and after major arterial occlusion [49]. The expression of latent and active forms of MMP-2 and –9 are shown in Figure **13**. MMP-2 expression was significantly elevated in collaterals at days 4 and 7 and percent of the activated form increased ~4X by day 4. No difference in MMP-9 expression between controls and collaterals was detected during the first week after chronic flow elevation. The increase in MMP-2 was confirmed by immunofluorescence (Fig. **14**) which also demonstrated increased expression of the MT1-MMP, a key physiological regulator of MMP-2. The increases in MMP-2 and MT1-MMP primarily occurred in the media, where increases in phosphorylated c-Jun and Egr-1(transcriptional activators of MMP-2 and MT1-MMP, respectively) were also observed [49]. Treatment with the general MMP inhibitor doxycycline suppressed MMP-2 expression and activation and prevented significant increases in luminal diameter, but not medial area or intimal cell number.

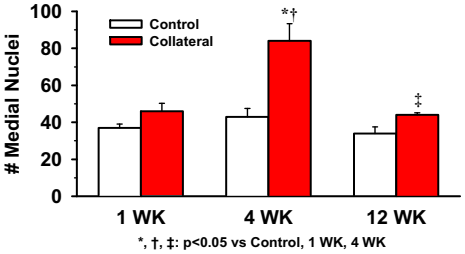

Figure 12: Number of medial nuclei in cross-sections of control and collateral arteries at 1, 4, and 12 weeks after abrupt flow elevation by occlusion of adjacent arteries. Data from Unthank et al. [2,46].

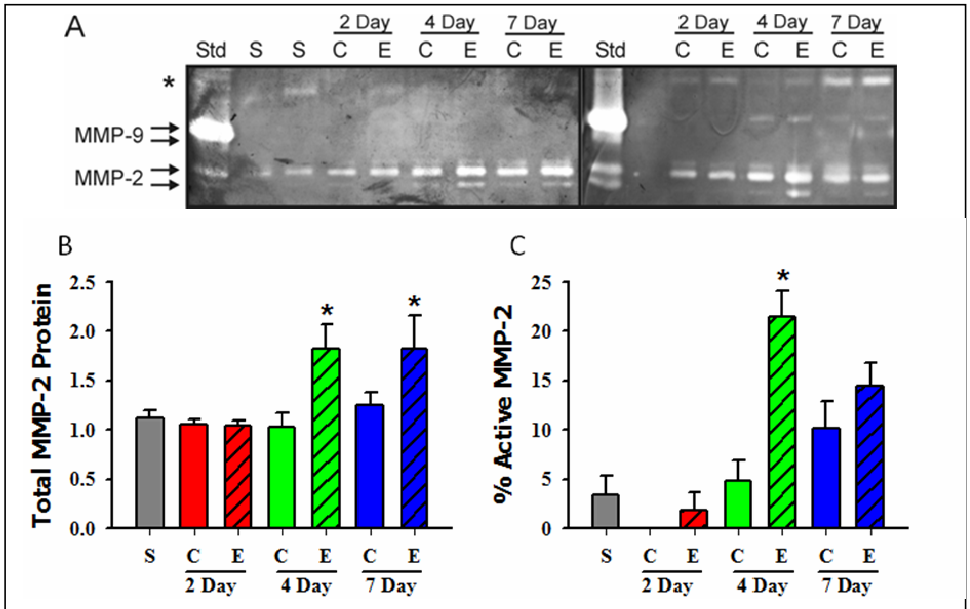

Figure 13: Expression of MMP-2 and MMP-9. Arteries were harvested at 2, 4, or 7 days after ligation surgery and MMP expression analyzed by zymography. A: representative gelatin zymographs showing MMP-2 to be present in latent (72-kDa) and active (62-kDa) forms and MMP-9 present in latent (92-kDa) form and in high-molecular-weight form corresponding to dimerized latent MMP-9 (*). S, sham operated; C, control artery; E, experimental collateral artery. B: densitometric analysis of MMP-2 protein shows total MMP-2 protein to be significantly increased in collateral compared with time-matched control arteries at 4 and 7 days. C: percentage of MMP-2 in active (62-kDa) form 2, 4, and 7 days after ligation surgery. Percentage of active MMP-2 increased significantly compared with control arteries at 4 days. *: $P \leq 0.05$ vs. time-matched control ($n = 4$). From Haas et al. [49].

In a different rat mesenteric model, Dumont et al. [50] observed an increase in MMP-9 but not MMP-2 expression at 4 days after abrupt flow elevation. Expression was assessed by immunostaining rather than gelatin zymography and the percent of activation was not evaluated. The magnitude of flow and shear stress elevation were not reported. In larger carotid arteries where flow and shear stress were increased to levels comparable to our model by creation of an arteriovenous fistula, Tronc et al [51] observed an increase in the active form of MMP-2 but not MMP-9, consistent with our observation.

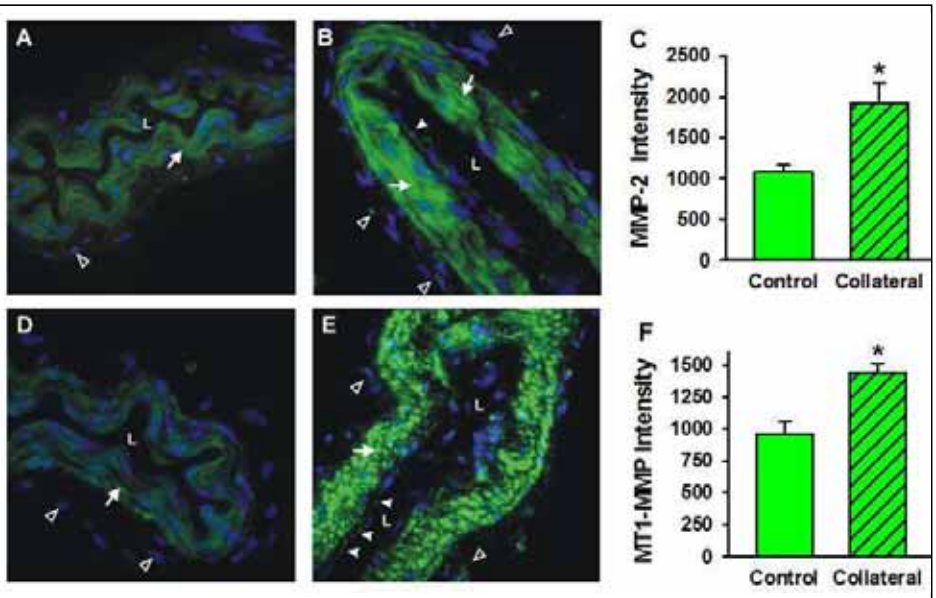

Figure 14: Immunolocalization of MMP-2 and MT1-MMP in control and collateral arteries. Representative images are shown for control (A and D) and collateral (B and E) arteries immunostained for MMP-2 (A and B) or MT1-MMP (D and E) 4 days after ligation. Sections were counterstained with diamidino-2-phenylindole (DAPI, blue) to visualize nuclei. A: diffuse staining of MMP-2 within smooth muscle layers of control arteries. B: intense staining of MMP-2 in collateral arteries. Immunostaining appeared predominantly in smooth muscle layers, with intermittent endothelial staining. D: weak staining of MT1-MMP, the activator of MMP-2, in control arteries. E: in collateral arteries, localization of MT1-MMP, with intense staining, to smooth muscle layer, with intermittent labeling of endothelial cell layer and adventitia. Arrows, staining within smooth muscle layers; solid arrowheads, endothelial cell staining; open arrowheads, adventitial cells. L, lumen. Magnification 400X. C and F: pixel intensities (arbitrary units) for MMP-2 and MT1-MMP. *: $P \leq 0.05$ vs. control. From Haas et al. [49].

Impact of shear level on wall areas, cellular remodeling, rate of expansion, and eNOS expression

The above data suggested to us that a number of events occur transiently rather than progressively throughout the period of luminal expansion. MMP-2 production and activation, endothelial cell proliferation/recruitment, and medial layer expansion due to smooth muscle cell hypertrophy occur during the first week following model creation when shear forces are at their highest. As the shear level is returned toward normal levels, between 1 and 4 wk, the rate of luminal expansion is decreased and smooth muscle cell hyperplasia is observed [46,48]. These observations led to the formulation of two hypotheses: 1) both the rate and magnitude of luminal expansion are dependent on the degree of initial shear elevation, and 2) specific remodeling events within the arterial wall layers occur in a temporal sequence dependent upon the level of shear alteration. Based upon the studies

available at the time [7,42], we presumed the shear-related events were also linked to increased eNOS expression and NO production.

We obtained initial results related to the impact of wall shear level on cell proliferation in a simple modification of our model [52]. However, the interpretation of this data was confounded by the involvement of different branching orders. To more clearly investigate the effect of the magnitude of change in blood flow and wall shear force with abrupt occlusion, we developed a new model [53] as shown in Fig. **15**. Ligations were selectively placed on ileal and marginal arteries to vary the length of bowel and number of microvascular units perfused by individual mesenteric arteries to ~50, 200, and 400% of their control condition as illustrated. With this approach, we were able to create three levels of altered arterial blood flows and shear forces in identical branching order arteries within the same animal as shown in Figs. **15** and **16**. Luminal dimensions, wall areas, nuclear densities, and endothelial nitric oxide synthase (eNOS) expression were measured to investigate how the level of altered shear impacts the rate of remodeling, wall expansion and composition, and endothelial gene expression.

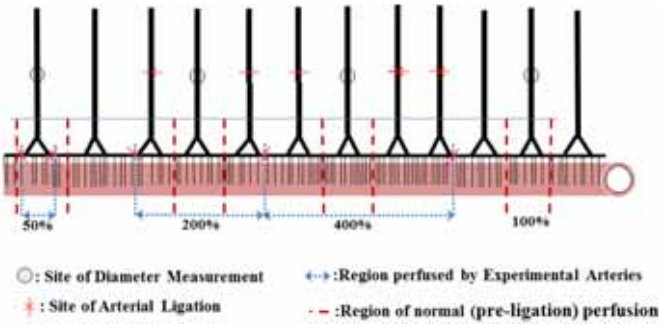

Figure 15: Schematic of multiple shear level mesenteric model. The normal perfusion territory for the arteries of interest was assumed to be the mid-point between it and the adjacent artery on each side as indicated by vertical dashed lines. This perfusion territory was reduced by 50% or increased to 200% or 400% of the original by ligations placed on ileal and marginal arteries. Altered perfusion territories are shown by blue dotted horizontal lines with arrows. Modified from Tuttle et al. [53].

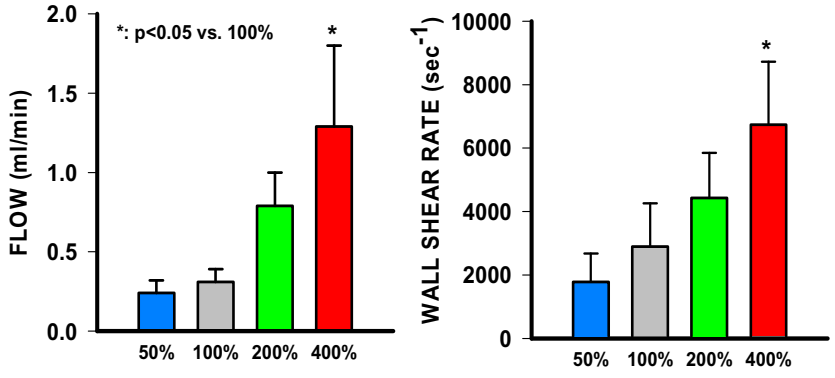

Figure 16: Arterial flow and wall shear rate for the different vessel types from Fig. **15**. A significant linear relationship existed between prefusion territory and both flow and wall shear rate. From Tuttle et al. [53].

Impact of shear level on magnitude and rate of luminal expansion

Table 1. Arterial Diameters

Vessel Type	2 Day Group		7 Day Group	
	D0	D2	D0	D7
50%	383±7.9	348±8.6*	377±15.6	339±16.1*
100%	350±10.2	357±6.2	342±14.5	348±11.3
200%	352±11.4	359±8.5	341±12.3	397±12.3*
400%	368±14.3	402±14.4*	346±14.9	457±18.0*

Values are means ± SE (in μm) for the 50%, 100%, 200%, and 400% vessel types for the 2-day group (n = 7) and the 7-day group (n = 10). *$P \leq 0.05$ vs. D0 within vessel type. D0, Day 0; D2, Day 2; D7, Day 7. From Tuttle et al. [53].

In vivo diameters of the various vessel types obtained under maximally dilated conditions are reported in Table 1. Diameters of all vessel types were similar at the time of model creation (D0). No change occurred in the diameter of the control (100%) arteries at either 2 or 7 days (D2, D7) after arterial ligation. The 50% arteries had a reduced luminal diameter at 2 days which was not further decreased at 1 wk. Consequently, the greatest rate of change in diameter of the 50% arteries occurred during the first 2 days (Fig. **17**). The highest flow (400%) arteries also were characterized by a significant diameter change at 2 days and the rate of diameter change was similar from D0 to D2 and D0 to D7 (Fig. **17**). The 200% arteries did not experience a significant increase in diameter until day 7 (Table 1), and the rate of diameter change in these vessels was greater after day 2 (Fig. **17**). Both the magnitude (Table 1) and the rate (Fig. **17**) of diameter change were greater in the 400% than 200% vessels.

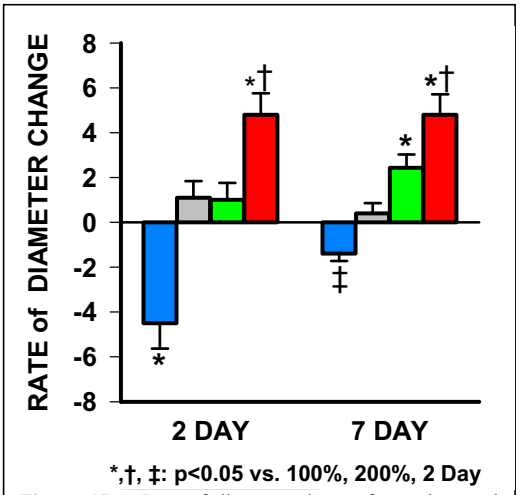

Figure 17: Rate of diameter change for each vessel type calculated as percent change in diameter divided by number of days after model creation. Blue, gray, green, and red bars represent vessel types 50, 100, 200, and 400%, respectively. From Tuttle et al. [53].

It is interesting to note that while the magnitude of the change in flow and wall shear were similar in the 50% and 200% vessels (Fig. **16**); the rate of change at day 2 was much greater in the 50% vessels. This could indicate that resistance vessels are more sensitive to and respond more rapidly to decreases in wall shear forces than increases as observed by in rabbit carotid arteries [54]. This may explain the rapid regression of collaterals after installation of a bypass graft or stent [55-56].

Impact of shear level on wall areas and nuclear densities

Wall areas were determined from arterial cross-sections as in previous studies. At 2 and 7 days post-occlusion, intimal and medial areas (Fig. **18**) were altered from control arteries only in the 400% arteries where they were increased. Brownlee and Langille [54] reported that a 60% increase in carotid artery flow did not result in luminal or medial expansion in mature rabbits and proposed that a threshold may exist for shear stress below which the vessels are unresponsive. Langille [14,57] also observed that with inward carotid artery remodeling associated with a reduction in flow, no change occurred in the major wall components.

Cell densities were determined from whole vessel imaging of intimal, medial, and adventitial nuclei as developed by Arribas and McGrath [58-59]. Intimal and adventitial cell densities (Fig. **19**) were only altered in the 400% where they were increased. Immunostaining for the cell proliferation marker Ki-67 demonstrated significant nuclear reactivity at day 2 (Fig. **20**), suggesting that the increase in intimal and adventitial cell densities were primarily due to proliferation rather than recruitment. Sho et al. [48] observed a similar endothelial response in carotid arteries exposed to elevated flow. Their kinetic analysis indicated that the

increased endothelial cell density was primarily mediated by a proliferative response which mostly occurred before 3 days [48]. Consistent with the preservation of cell density in reduced flow vessels, Langille et al. [14] demonstrated that endothelial cell density is preserved by cell loss during inward remodeling associated with reduced flow in carotid arteries of mature rabbits.

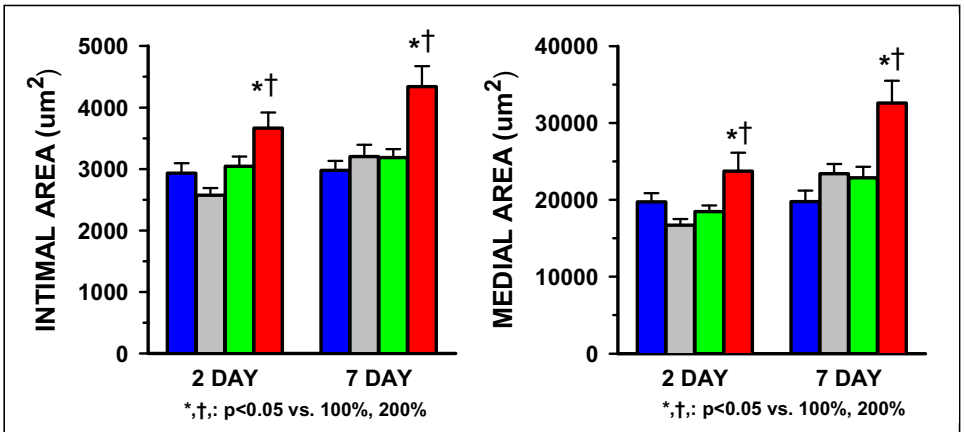

Figure 18: Intimal and medial cross-sectional area for each vessel type. Blue, gray, green, and red bars represent vessel types 50, 100, 200, and 400%, respectively. Significant increases were observed only in the 400% vessels. From Tuttle et al. [53].

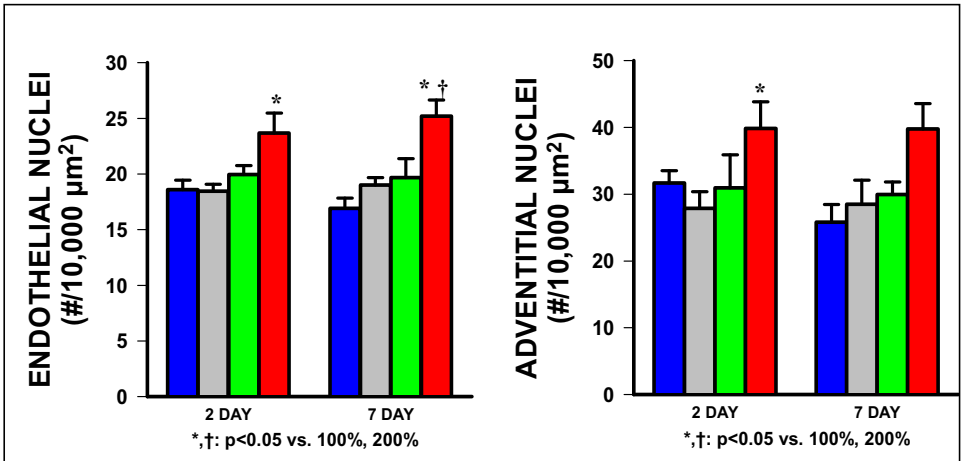

Figure 19: Number of intimal and adventitial cells per unit area determined from whole vessel imaging. Blue, gray, green, and red bars represent vessel types 50, 100, 200, and 400%, respectively. Significant increases were observed only in the 400% vessels. From Tuttle et al. [53].

It is important to note that while increases in wall areas and cell densities in the 400% arteries were already apparent at 2 days (Fig. **18** and **19**) when luminal diameter had only increased about 10% (Table 1), no increases had occurred by 7 days in the 200% arteries which had a luminal expansion of 17%. Also, wall areas and nuclear densities were not altered in the 50%

vessels which had a rate of expansion similar in magnitude to the 400% arteries. Whereas previous studies have demonstrated that changes in wall areas and cell densities are altered by an increase or decrease in blood flow, this was the first study to demonstrate that these specific remodeling events associated with flow-mediated luminal expansion are dependent upon the initial stimulus level. With either a reduction or moderate elevation in flow the remodeling was eutrophic in nature, but hypertrophic in the vessels experiencing greater flow elevation. These data suggest that enlarging collateral vessels located in the same animal could exhibit markedly different histological and morphometric characteristics depending upon the specific hemodynamic stimuli to which they are exposed.

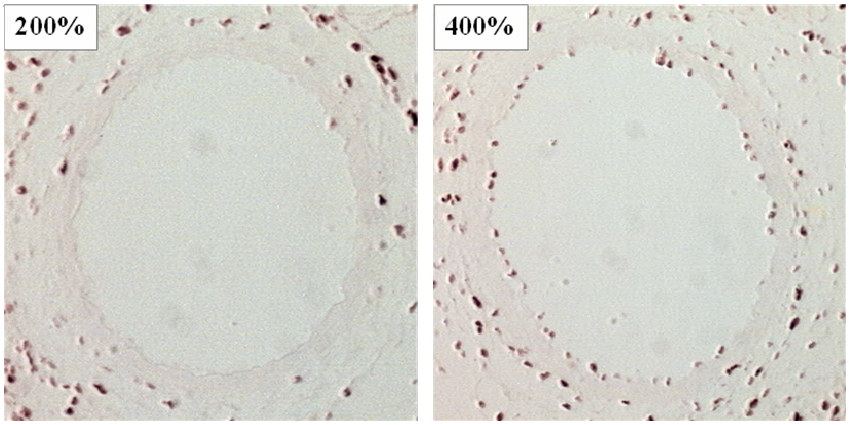

Figure 20: Representative micrographs of 200% and 400% arteries with immunohistochemical staining for the cell proliferation marker Ki-67 at 2 days after arterial ligation. The results are consistent with the increase in intimal and adventitial cell density observed in the 400% but not 200% vessels and indicate a proliferative response is primarily responsible.

Effect of shear level on endothelial gene expression

Because studies had suggested that increased eNOS expression was associated with successful flow-mediated luminal expansion [7] and that NOS inhibition prevented flow-mediated outward remodeling [42], we evaluated eNOS expression in the various arteries by quantitative immunohistochemistry. eNOS staining was present only within the endothelium and varied with flow condition as illustrated in Fig. **21**. eNOS content normalized to luminal circumference was greater in the 400% than in the 100% arteries at both day 2 and day 7. Assessment of eNOS expression by Western blotting in 100% and 400% arteries indicated a 3.7 fold increase at day 2 [53]. As this increase in eNOS content was much greater than the increase in endothelial cell density (~30%), the increased eNOS content is primarily the result of increased cellular expression rather than elevated cellular density.

Studies by others using a similar mesenteric model confirm our results demonstrating increased eNOS expression and provide additional evidence of the importance of eNOS derived NO in the flow-mediated response. eNOS expression determined by Western blotting was increased ~50-100% from 4-7 days after flow elevation [50,60]. Both studies demonstrated the suppression of flow-mediated outward remodeling by the NOS inhibitor L-NAME in rat mesenteric arteries. In addition, Dumont et al. [50] observed suppression of outward but not inward flow-mediated remodeling in mesenteric arteries of eNOS deficient mice. However, not all studies are consistent with a significant role for eNOS derived NO in flow-mediated outward remodeling. L-NAME inhibits all NOS isoforms and Mees et al. [61] have presented data suggesting that collateral development in the mouse hindlimb is dependent upon iNOS rather than eNOS.

Together, our data from the multiple shear level model provide compelling support for the hypothesis that specific wall remodeling events, including the magnitude and rate of luminal expansion, cross-sectional wall areas, and cell proliferation, are dependent on the level of the shear stimulus.

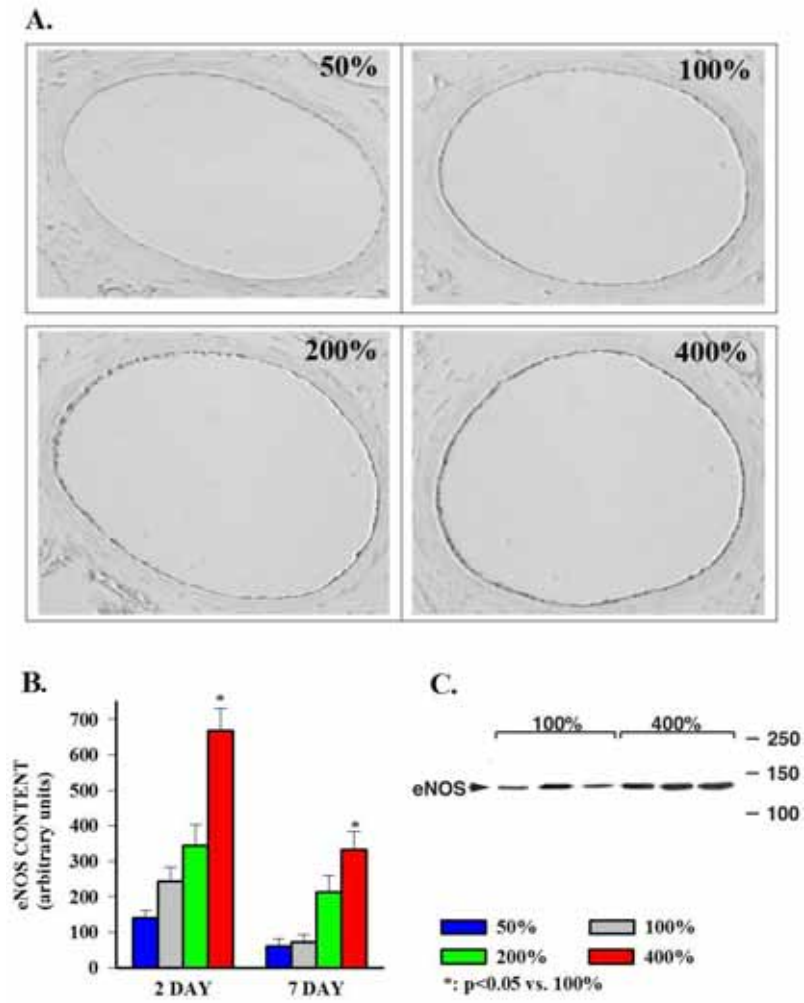

Figure 21: Endothelial nitric oxide synthase (eNOS) staining and average content in mesenteric arteries (n = 8) are shown in the micrographs (A, representative images from the 2-day group) and plot (B). Specific eNOS staining is localized to the endothelium within the mesenteric arteries. A significant increase in eNOS (*B*) content was observed between the 400% and 100% arteries both 2 and 7 days postligation and was confirmed with Western blotting (*C*). From Tuttle et al. [53].

Impact of risk factors on flow-mediated collateral growth

It has been known since the last century that advancing age and vascular risk factors suppress the capacity of the human circulation to compensate for abrupt occlusion of a major

peripheral artery (see review by Ziegler et al. [15]). Animal studies also support this observation regarding collateral growth and even juvenile maturation can impact the capacity for flow-mediated remodeling [7,54]. Yet few investigations have addressed the specific abnormalities and potential mechanisms until recently. Vascular remodeling is a complex process and impairments could result from one or more deficiencies in signal transduction; transcription factor activation; gene transcription and translation; cellular proliferation, growth and recruitment; and abnormal expression or activation of receptors and matrix metalloproteases and their inhibitors. Having completed the above studies in young healthy animals, we undertook the investigation of mechanisms involved in collateral growth impairment which occurs in the presence of risk factors. We performed studies in both the spontaneously hypertensive rat (SHR) and in aged, normotensive rats. The SHR is a genetic model of essential hypertension and is also characterized by endothelial dysfunction, elevated oxidative stress, an activated renin angiotensin system, and metabolic abnormalities. Multiple studies with SHR have documented suppressed compensation to arterial occlusion [62-74]. The aged rats used were retired breeders ≥8 months of age. This age of rat is typically characterized by elevated oxidative stress and endothelial dysfunction [75-77] and even much younger rats have been shown to exhibit impaired remodeling responses to chronically elevated blood flow [7]. Clinical studies have shown that collateral growth is impaired in the presence of risk factors for vascular disease, including hypertension [78-83]. Based upon these clinical studies, and evidence that NO and eNOS were important for flow-mediated remodeling in large conduit arteries [7, 42], and that shear level and presumably NO levels influenced specific remodeling events, we hypothesized that chronic oxidative stress impaired collateral growth through reduced NO bioavailability.

SHR-Impairment of flow-related remodeling reversed by anti-oxidant therapy, requires intact NOS system

Nature of impaired flow-mediated remodeling. Our studies in the SHR mesentery demonstrated that, despite a comparable increase in blood flow and wall shear rate in the experimental collateral arteries relative to WKY, luminal expansion was significantly suppressed [74]. Cross-sectional wall areas and nuclear numbers for control and collateral arteries in SHR and their normotensive controls are reported in Table 2. Other than the reduced luminal expansion in SHR, the most notable difference was a suppressed intimal response. Compared to same animal control arteries, the number of collateral intimal cell nuclei was increased 80% ± 23% in WKY but only 22% ± 7% in SHR [74]. Another major difference between SHR and WKY experimental collateral vessels was the lack of eNOS upregulation in the SHR (Fig. **22**). These two endothelial abnormalities may have contributed to the impaired response observed in the SHR collaterals.

Table 2. Measurements from arterial cross-sections

	WKY control	WKY collateral	SHR control	SHR collateral
Luminal area (μm^2)	43,500 ± 11,200	68,900 ± 6400*	31,900 ± 4560	40,800 ± 3600*†
Medial area (μm^2)	19,000 ± 3550	25,500 ± 2670	23,200 ± 1260	29,700 ± 2050†
#Medial nuclei	31 ± 6	30 ± 2	37 ± 2	39 ± 3†
#Intimal nuclei	20 ± 2	35 ± 3*	28 ± 2†	35 ± 3*

*, †:P≥ 0.05 collateral vs control, SHR vs WKY. From Tuttle et al.[74].

Reversal by anti-oxidant therapy. In seeking to identify potential mechanisms that might mediate the impairment of flow-mediated outward remodeling in SHR mesenteric arteries, we became aware that angiotensin converting enzyme inhibitors (ACEI) had been shown to promote collateral growth in both hypertensive humans [84] and animals [65]. In the SHR hindlimb, chronic administration of the ACEI ramipril increases tissue perfusion without altering skeletal muscle capillary or arteriole density [65], suggesting that its effect was mediated by collateral growth. Neither the clinical nor preclinical study identified the specific mechanisms responsible for the perfusion impairment or its reversal by ACEI. Because ACEI are known to improve endothelial function, we performed pilot studies with the ACEI

captopril, and observed a remarkable enhancement of collateral growth in the SHR [73]. Additional experiments were then performed to investigate the hypothesis that SHR collateral growth impairment is mediated by excess superoxide produced by NAD(P)H oxidase in response to stimulation of the angiogensin II type 1 receptor (AT1R) by angiotensin II (AngII) [73]. As illustrated in Fig. **23**, our results demonstrated that SHR collateral growth was enhanced by captopril, the superoxide dismutase mimetic tempol, and the anti-oxidant and NAD(P)H oxidase inhibitor apocynin, but not by the AT1R antagonist losartan or the ACEI enalapril. The effects of apocynin and tempol were observed to be dose dependent and the beneficial effects were independent of pressure changes [73]. Because captopril has anti-oxidant effects due to its sulfhydryl group [85] independent of its effects on AngII and activation of the AT1R, the data are most consistent with a beneficial effect of anti-oxidants. The combination of low dose L-NAME with captopril greatly attenuated the response; providing additional supporting evidence for an important role for NO.

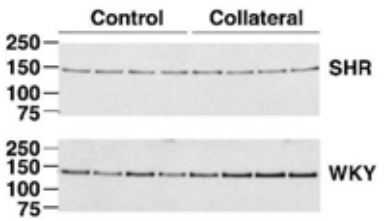

Figure 22: Representative Western gel for protein extracts from four control and collateral arteries of both SHR and WKY 2 days after model creation. The level of eNOS protein was greater in collateral relative to same animal control arteries in WKY but not SHR, with the average collateral/control ratio being 2.28 ± 0.58 in WKY and 1.17 ± 0.06 in SHR. Figure from Tuttle et al. [74].

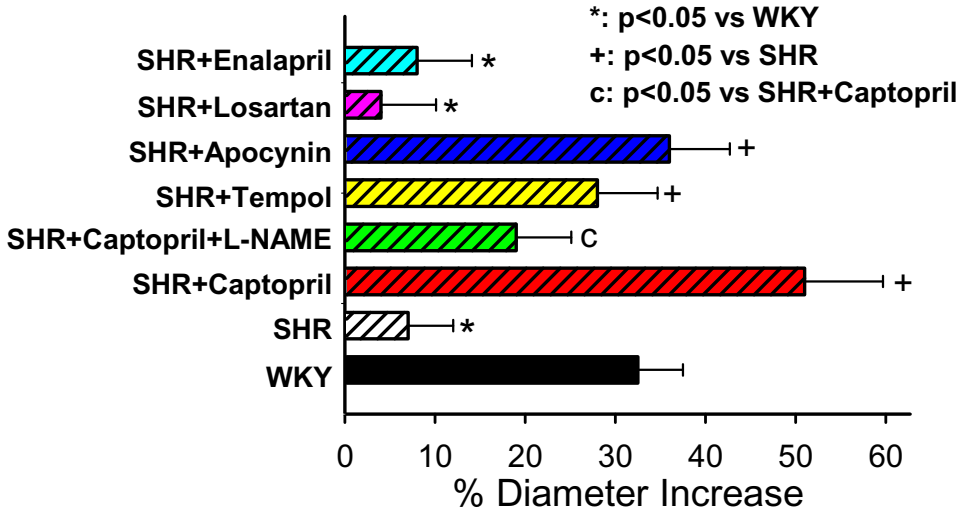

Figure 23: Percent increase in mesenteric collateral artery diameter for untreated WKY and SHR, and various treatments in SHR. Data from Miller et al. [73].

Retired breeder rats-impairment of flow-related remodeling reversed by anti-oxidant therapy, requires intact NOS system.

We performed additional studies [77,86] to investigate the effect of aging on flow-mediated remodeling and collateral luminal expansion in Wistar and WKY retired breeder (RB) rats, ≥8 months of age.

Nature of impaired flow-mediated remodeling and luminal expansion. As illustrated in Figure **24**, the maximally dilated in vivo diameter of collateral arteries one week after arterial occlusion was significantly increased in young (33±7%) but not Wistar RB rats (1±5%). No

significant changes occurred in the diameters of control arteries. Morphometric analyses of arterial cross-sections revealed that the number of endothelial nuclei were significantly increased in both young and retired breeder rats (Fig. **25**). Cross-sectional medial area was also increased in both age groups (Fig. **26**).

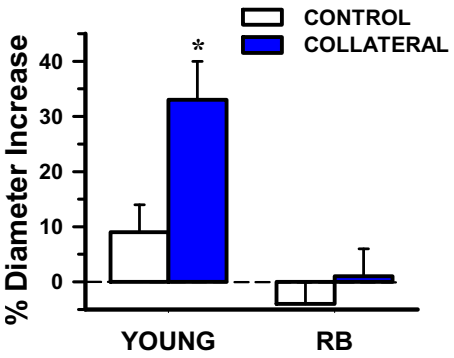

Figure 24: Percent diameter change in control and collateral arteries of young and retired breeder (RB) Wistar rats at 7 days after model creation From Tuttle et al. [86].

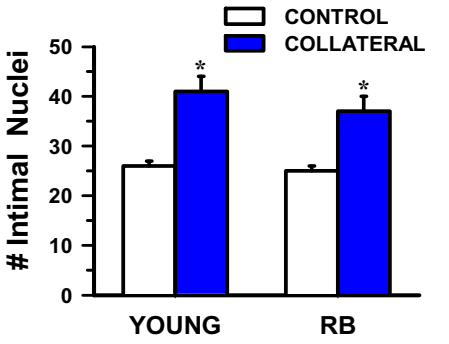

Figure 25: Number of intimal (endothelial) cell nuclei in cross-sections of control and collateral arteries from young and retired breeder (RB) Wistar rats at 7 days after flow elevation by arterial ligation. From Tuttle et al.[86].

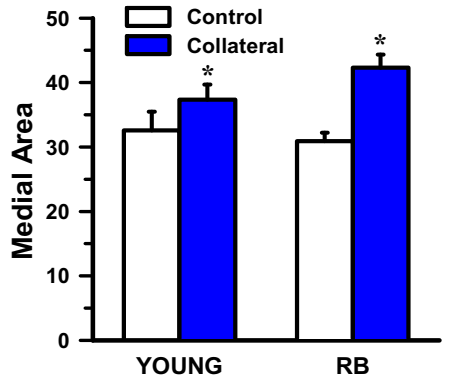

Figure 26: Medial cross-sectional area (x1000 μm^2) of control and collateral arteries from young and retired breeder (RB) Wistar rats at 7 days after flow elevation by arterial ligation. From Tuttle et al. [86].

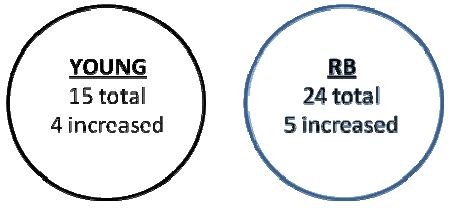

Figure 27: When protein expression patterns of control to collateral arteries were compared, 15 arterial proteins differed in abundance (4 higher and 11 lower) in the young animals, whereas 24 (5 higher and 19 lower) proteins had differing abundances in the mature animals. There was no overlap in the proteins displaying altered expression in response to elevated flow (collaterals) between young and mature rats; none of the 15 proteins displaying altered expression in young rats exhibited altered expression in mature animals. From Tuttle et al. [86].

Molecular basis of impairment. Proteomic analysis was performed to obtain insight into potential molecular mechanisms that might mediate the flow-mediated remodeling impairment observed in Wistar RB rats [86]. Control and collateral mesenteric arteries were harvested from each animal. Proteomic analyses were performed using two-dimensional electrophoresis and peptide mass fingerprinting. Approximately 1200 protein spots were detected and matched in sample gel patterns. Protein spots having altered expression between same animal collaterals and controls were identified. While a similar number had increased and decreased expression between young and retired breeder rats, a fundamentally different expression pattern was observed as illustrated in Figure **27**. None of the proteins

with altered collateral expression were similar between young and RB. Proteins with increased collateral expression in young Wistars included vimentin, and heat shock proteins 27 and 70. The global difference in collateral protein expression between retired breeder and healthy young rats is consistent with the concept that redox status regulates gene expression in the vasculature [87-88] and impacts pathological as well as compensatory remodeling.

Anti-oxidant therapy reverses age-related collateral growth impairment and requires intact NOS system. Subsequent studies were performed to determine if anti-oxidant therapy could reverse the age-related impairment of collateral growth as observed in SHR [77]. As shown in Figure **28**, there was no increase in collateral diameter measured under maximally dilated conditions 1 week after model creation in Wistar RB rats. Pre-treatment with tempol restored the capacity for collateral growth as evidenced by the ~20% diameter increase. This effect of tempol was prevented when administered in combination with low-dose L-NAME. Figure **29** demonstrates that the anti-oxidant apocynin has effects similar to tempol in Wistar and WKY RB when administered both before and after occlusion.

While the effects of apocynin and tempol when administered prior to model creation provide important evidence that the mechanisms mediating age-related collateral growth impairment are dependent upon increased oxidative stress, the similar effects observed with post-treatment have clinical relevance as they suggest that aggressive anti-oxidant therapy may provide benefit to human with existing occlusive disease.

The effects of anti-oxidant therapies on collateral growth in both SHR and retired breeders are consistent with the hypothesis that elevated oxidative stress impairs flow-mediated collateral growth. The observation that low dose L-NAME prevents the beneficial effects of the anti-oxidants also suggests that the mechanism of impairment is related to bioavailable NO or flow-mediated NO production. Vita et al. have recently shown a correlation between the capacity for acute flow-mediated dilation and flow-mediated remodeling in the ulnar artery in humans [10]. Based upon a strong negative correlation between P-selectin immunoreactivity and flow-mediated outward remodeling, they speculated that the existence of a pro-inflammatory state prior to elevated blood flow prevents outward remodeling. Since a pro-inflammatory state and impaired flow-mediated dilation are associated with oxidative stress, the reversal of oxidative stress and restoration of endothelial function may restore and enhance the capacity for flow-mediated remodeling and collateral growth. However, existing studies also suggest that a certain level of ROS are critical for flow-mediated remodeling in mesenteric arteries [89] and for compensation for vascular compensation to repetitive coronary artery occlusion [90]. Thus, additional experimentation is warranted to investigate the specific ROS involved in both the promotion and impairment of flow-mediated remodeling including collateral growth.

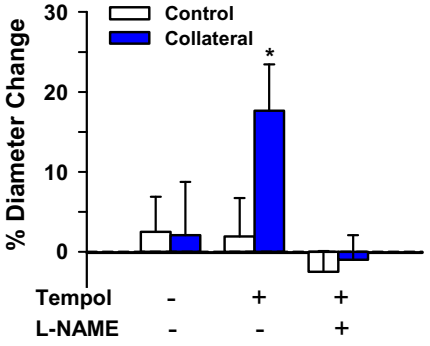

Figure 28: Percent diameter change 7 days after model creation in control and collateral arteries of retired breeder Wistar rats receiving no treatment or tempol with and without L-NAME. From Miller et al. [77].

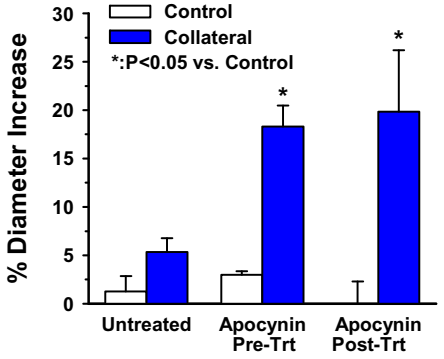

Figure 29: Percent diameter increase in control and collateral arteries of Wistar and WKY RB rats receiving no treatment, apocynin before (Pre-trt), or 1 week after (Post-trt) model creation. Data from Miller et al. [77].

Impact of oxidative stress on bioavailable NO in mesenteric arteries

Our studies described above are consistent with the hypothesis that a normally functioning eNOS system is required for flow-mediated remodeling and collateral growth and that impairment of these processes occur when elevated superoxide reduces NO bioavailability. While numerous studies with NOS inhibition and ablation have suggested NO and eNOS to be important, if not required, for flow-mediated remodeling and compensation to arterial occlusion, contradictory studies also exist [61,91]. Interpretation of results are confounded by studies which have shown that NOS inhibition can impact arterial remodeling by inducing oxidative stress and inflammation [92-93] and that vascular compensation occurs in eNOS knockout mice to maintain dilatory responses to flow-related stimuli [94-95]. Our studies, as well as others which have shown increased eNOS expression in enlarging collaterals, cannot be used to establish a definitive role for NO as these studies have not distinguished between membrane bound and total cellular eNOS protein, determined eNOS activity or phosphorylation, or evaluated eNOS uncoupling and superoxide production. To complicate the issue even more, an NO-resistant state that can be reversed by anti-oxidant therapy has recently been described in diseased blood vessels from humans and animals [96-97]. The presence of an NO resistance state is consistent with studies in SHR and aged rats that have shown abnormalities in cGMP signaling mechanisms which include reduced sGC expression [98-101]. Thus, it is certainly possible that the beneficial effects of anti-oxidant therapy used in our studies of impaired mesenteric collateral growth involve mechanisms other than bioavailable NO.

To begin to address these issues, we have made direct, in vivo measurements of per-arterial NO and H_2O_2 in young WKY, SHR [102] and WKY-RB [76]. The results described below were significantly different than we had expected and have revolutionized our perspective of the mechanisms responsible for collateral growth impairment.

Model and experimental approach**.** Our model was modified as shown in Figure **30** to permit measurements during step changes in blood flow (Fig. **31**).

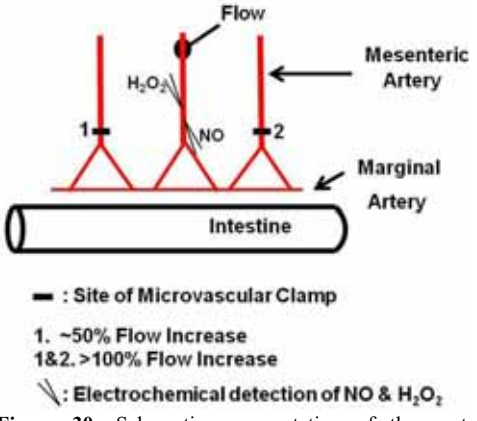

Figure 30: Schematic representation of the acute model. Our experimental model was modified to utilize a standard chamber for intravital microvascular studies. Flow, NO and H_2O_2 were measured simultaneously at sites illustrated before and after temporary occlusion of adjacent arteries to elevate flow. The application of microvascular clamps to the adjacent arteries allowed flow to be elevated in a linear and stepwise manner as shown in Fig. **31**.

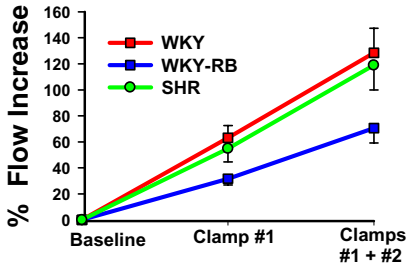

Figure 31: Relationship between flow and clamp condition for WKY, SHR, and WKY-RB. NO and H_2O_2 were measured at control (baseline) and after placement of 1 and 2 microvascular clamps on adjacent arteries. In this review, only the combined clamp data is presented. From Zhou et al. [76, 102].

Peri-arterial NO is elevated in SHR and retired breeder-WKY and suppressed by NAD(P)H oxidase inhibitors. To our surprise, peri-arterial [NO] under baseline in vivo conditions in SHR and WKY-RB was not reduced but substantially increased (Fig. **32**). Furthermore, pre-treatment with the anti-oxidant apocynin, as performed in earlier studies and which reversed collateral growth impairment, reduced rather than increased baseline [NO] in both SHR and

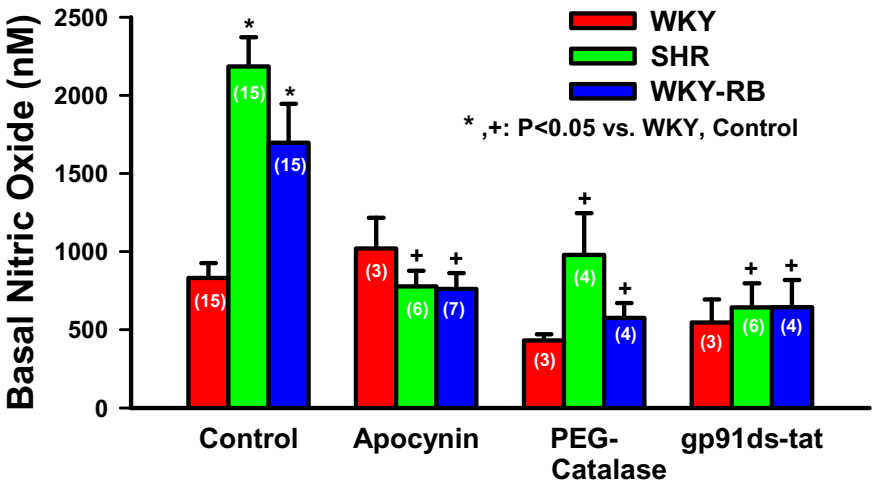

Figure 32: Direct in vivo measurement of periarterial NO with electrodes. Under control conditions, NO is elevated 2-3X the WKY level in SHR and retired breeder WKY (RB-WKY). Apocynin pre-treatment and topical PEG-catalase and gp91ds-tat decrease the NO to the concentration observed in untreated WKY. Sample size is indicated by the number in parentheses in the column bar. From Zhou et al. [76, 102].

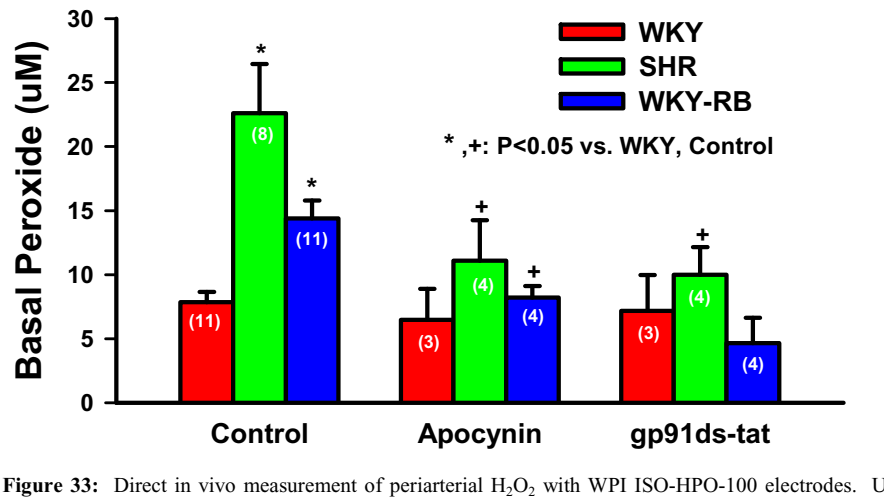

Figure 33: Direct in vivo measurement of periarterial H_2O_2 with WPI ISO-HPO-100 electrodes. Under control conditions, H_2O_2 is elevated 2-3X WKY level in SHR and retired breeder WKY (RB-WKY). Apocynin pre-treatment and topical gp91ds-tat decrease the H_2O_2 to the concentration observed in untreated WKY. Sample size is indicated by the number in parentheses in the column bar. From Zhou et al. [76, 102].

WKY-RB. As we considered explanations, we found several reports of increased eNOS activity and NO production in SHR [103-106] and eNOS expression in aged [107-108] rats. In vitro studies have also indicated that increased NAD(P)H oxidase activity elevates NO production from endothelial cells via H_2O_2-dependent activation of Akt and ERK1/2 [109-110]. Additional experiments with the cell permeable form of catalase (polyethylene glycated, PEG-catalase) and the specific NAD(P)H oxidase inhibitor, gp91ds-tat, produced results similar to those of apocynin. Together, these results suggest that the increased bioavailable NO in SHR and WKY-RB under in vivo conditions is due to the stimulation of eNOS by hydrogen peroxide derived from NAD(P)H oxidase.

Peri-arterial H_2O_2 elevated in SHR and retired breeder-WKY and suppressed by NAD(P)H oxidase inhibitors and PEG-catalase. Measurement of peri-arterial H_2O_2 demonstrated a significant increase in SHR and WKY-RB relative to young WKY (Fig. **33**). Pretreatment with apocynin and acute application of gp91ds-tat significantly decreased [H_2O_2] to levels measured in young WKY. The elevation of vascular H_2O_2 in SHR and aged animals is consistent with previous studies showing increased ROS production and NAD(P)H oxidase expression/activity [75,111-114]. These studies are also consistent with the increased peroxide levels observed in the plasma of hypertensive humans [115-116] and the direct production of H_2O_2 from NAD(P)H oxidase [117].

Topical H_2O_2 increases peri-arterial NO in WKY but not SHR. To further evaluate the ability of H_2O_2 to increase NO production in vivo, topical H_2O_2 was added to young WKY and SHR (Fig. **34**). Addition of exogenous H_2O_2 to increase the suffusion solution [H_2O_2] to the level observed in SHR arteries at rest (20μM), increased [NO] in WKY arteries to the level observed in SHR. Further elevation of the bath [H_2O_2] resulted in an additional increase in WKY NO. In SHR, additional [H_2O_2] did not elevate NO. These data indicate that H_2O_2 is capable of elevating NO in vivo and suggest that this mechanism is maximally stimulated in the young SHR.

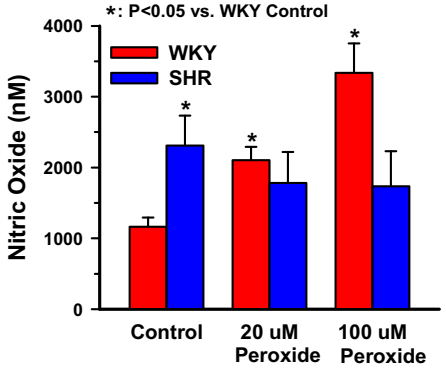

Figure 34: Effect of topical H_2O_2 on periarterial [NO] under normal flow conditions in young WKY and SHR. NO is increased in a concentration dependent manner by H_2O_2 in young WKY but not SHR. From Zhou et al. [102].

Flow-mediated NO production abolished in SHR and retired breeder-WKY and restored by NAD(P)H oxidase inhibitors and PEG-catalase. Under control conditions without any drug treatment, peri-arterial [NO] increased with flow only in young WKY. This is shown in Figure **35** for the highest level of flow elevation. Pre-treatment with apocynin, and acute topical application of PEG-Catalase and gp91ds-tat all restored the capacity for flow-mediated NO production in SHR and WKY-RB.

Abnormal flow-mediated H_2O_2 production observed in SHR and retired breeder-WKY and reversed by NAD(P)H oxidase inhibitors. Peri-arteriolar H_2O_2 was observed to increase with flow in SHR and WKY-RB, but not WKY. The percent increases under the highest flow conditions are shown in Figure **36**. Apocynin pre-treatment and acute application of gp91ds-tat prevented the flow-related elevation of H_2O_2. The increase in H_2O_2 with flow the SHR and WKY-RB is consistent with observations in diseased human arterioles [118-119].

The combined results with NO and H_2O_2 measurements in both young SHR and older adult WKY show that in vivo basal NO in mesenteric resistance arteries is not suppressed, but elevated, and the capacity for flow-mediated NO production is abolished. A critical role for peroxide is indicated by a) its elevation in SHR and RB-WKY, b) the reduction in basal NO and restoration of flow-mediated NO production by PEG-catalase, and c) the increase in NO with topical H_2O_2 in young WKY. In SHR and RB–WKY both apocynin and gp91ds-tat

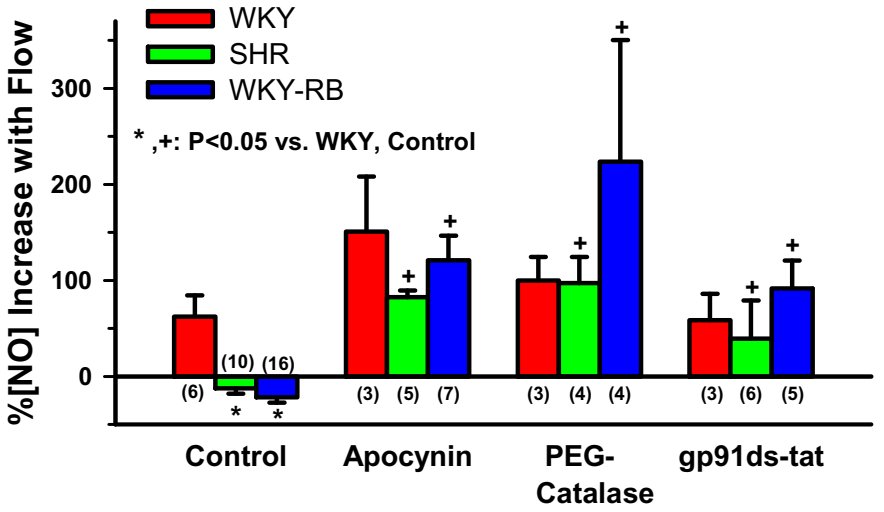

Figure 35: Effect of increased flow (2-clamp condition in Fig. **30**) on changes in peri-arterial [NO]. Under control conditions, NO was increased only in the young WKY. Apocynin pre-treatment and topical PEG-catalase and gp91ds-tat restored flow-mediated NO production in SHR and WKY retired breeder (WKY-RB). Sample size is indicated by the number in parentheses in the column bar. From Zhou et al. [76, 102].

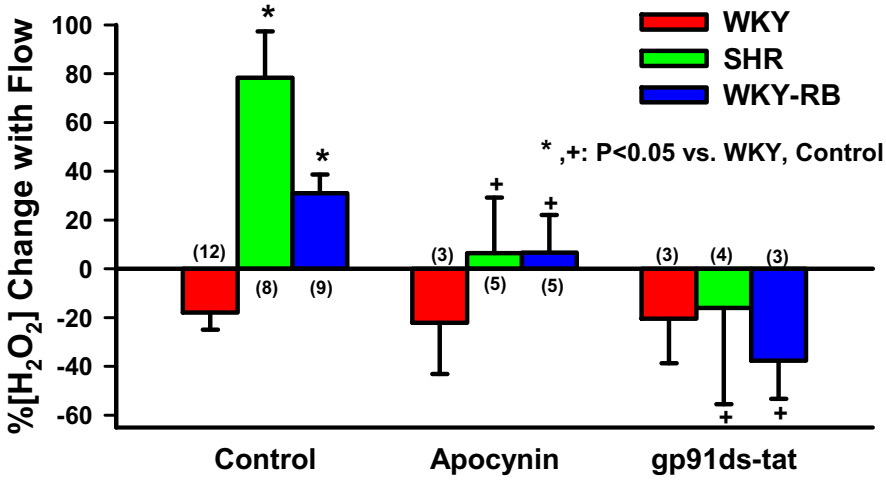

Figure 36: Effect of increased flow (2-clamp condition in Fig. **1**) on changes in peri-arterial [H_2O_2]. Under control conditions, H_2O_2 was increased with flow in SHR and WKY retired breeder (WKY-RB) but not young WKY. With apocynin pre-treatment and topical gp91ds-tat, the response in SHR and WKY-RB was similar to young WKY. Sample size is indicated by the number in parentheses in the column bar. From Zhou et al. [76, 102].

correct abnormalities in basal concentrations and flow-mediated production of NO and H_2O_2. These results provide evidence that in resistance vessels the major enzymatic source of H_2O_2 is NAD(P)H oxidase and also suggest that NAD(P)H oxidase-derived peroxide can exert a greater influence on NO levels than superoxide under in vivo conditions. The data are consistent with the hypothesis that a sub-phenotype of endothelial dysfunction exists which is characterized by high basal NO due to eNOS activation by NAD(P)H oxidase-derived H_2O_2.

This hypothesis is consistent with recent reports of elevated H_2O_2 and of NO insensitivity in humans and animals with arterial disease [96-101,115].

Summary and Conclusion

Hemodynamic forces, including the frictional wall forces associated with blood flow, are fundamentally important in regulating luminal diameter and wall structure in health and disease. We have utilized the mesenteric model to investigate cellular and molecular processes and mechanisms involved in successful flow-mediated remodeling of resistance vessels as well as the impaired remodeling that occurs in disease models characterized by oxidative stress. We have shown that specific remodeling events occur temporally rather than progressively during luminal expansion and that the specific level of flow or shear stimulus has a major impact on the rate and magnitude of remodeling as well as the associated cellular and molecular events. As shear stress influences both NO and ROS production within the vascular wall, and these reactive species influence all the major processes involved in vascular remodeling, it is likely that changes in their concentrations are fundamentally important in the specific remodeling events observed during luminal expansion. In the context of impaired flow-mediated luminal expansion, specific events like the intimal and medial responses proceed even without luminal expansion and the exact abnormality varies between models with different risk factors. Our data with direct in vivo measurement of NO and H_2O_2 indicate that the impairment of flow-mediated luminal expansion and remodeling in mesenteric arteries is not due to a reduction in bioavailable NO by superoxide scavenging as commonly thought. Rather, elevated H_2O_2 appears to increase [NO] through eNOS activation and may also induce an NO-insensitive state. Regardless, the ability of antioxidant therapies to reverse the impairment of flow-mediated mesenteric artery remodeling in hypertensive and retired breeder rats suggest that correction of a redox imbalance present with vascular risk factors represents a promising therapeutic approach to enhance the body's natural compensatory mechanisms. Recent clinical observations that endothelial dysfunction and a pro-inflammatory state are associated with impaired flow-mediated luminal expansion support this possibility. The development of an optimal anti-oxidant therapy will require the identification of the specific ROS and the enzymes that produce them in various disease states. The impact of potential therapies on peri-arterial [NO] and NO sensitivity also represent important areas for future studies. Because of its advantages, including direct in vivo measurement of ROS and NO, we believe the mesentery model for altering flow and the associated wall shear forces will have an important role in advancing our knowledge in these areas.

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Chapter 7. Epigenetic Mechanisms and Arteriogenesis

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Abstract: Epigenetics refers to chromatin-based pathways important in the regulation of gene expression due to mechanisms other than changes in DNA sequence. It includes distinct mechanisms, i.e. DNA methylation, histone posttranslational modifications as well as RNA–based mechanisms. Epigenetic processes have profound effects not only in the fine tuning of gene expression, but also concerning fundamental processes such as cell proliferation and differentiation. This chapter aims to provide insights into epigenetic mechanisms in terms of an overview; it also focuses on processes affecting in particular endothelial as well as vascular smooth muscle specific gene expression. Mechanisms of epigenetic processes are highlighted using in particular endothelial nitric oxide synthase (eNOS) gene expression as an example. Although currently no data are available *directly* demonstrating the influence of epigenetic modifications on the process of arteriogenesis, we attempt to illustrate the effects such modifications are likely to have on signal transduction cascades in arteriogenesis. Finally, we present data on bone marrow derived cells such as lymphocytes and monocytes and question the need for stem cells. In summary, this chapter aims to supply insight into epigenetics and epigenetic mechanisms, aiming to encourage scientists to accept the challenge to investigate the influence of epigenetic modifications on arteriogenesis and collaterogenesis.

Introduction

In the book *On the generation of animals,* Aristite postulated for the first time the view that during the embryonic development of an organism, structures are formed that are not preformed in semen or egg. The theory of epigenesis was born. The etymologic closely related biological term epigenetics was coined in 1942 by C. Waddington and today defines the "study of heritable changes in genome function that occur without a change in DNA sequence" [1].

Epigenetics is based on three pillars: DNA methylation [2], histone density and posttranslational modifications, and RNA-based mechanisms (Fig. **1**.) (for an overview see [3]. Epigenetic marks regulate the "open" or "closed" state for regions of the genome and thereby control the "on" and "off" status of a gene. Small interfering (si) RNAs as well as very big non-coding (nc) RNAs are the two epigenetic RNAs currently known. Whereas si RNAs are involved in establishing the "closed" configuration, the nc RNAs are known to be involved in both functions, establishing the "closed" as well as the "open" configuration. Histones forming the nucleosome can be modified at a number of sites e.g. by acetylation or methylation. Histone modifications can be read like a code and can determine whether a gene is switched "on" or "off" as well. The third component of epigenetic regulation is DNA methylation. Methylation of cytosine results in formation of "closed" chromatin and therefore in the switching "off" of genes. Epigenetic modifications can occur already during embryonic development, but also in adulthood, and are regulated by internal and external stimuli [4, 5]. The environmental factors influencing epigenetic marks are still poorly defined. However, it is clearly evident that even factors such as nutrition have an impact.

Generally, it appears that most genes are regulated epigenetically, and that the process presents, beside the activation of a transcription activator or -repressor, a second or rather upstream check point of gene expression. Although epigenetic marks are classed as heritable changes in the genome, not all epigenetic modifications are destined to be passed to the next generation. Epigenetic regulation can be established cell-autonomously through intercellular signaling elicited by environmental influences. All cells of a body are subjected to epigenetic regulation and all epigenetic processes are reversible. They are required for fine-tuning of gene expression and for fundamental cellular processes such as proliferation and differentiation. Thus understanding epigenetic regulation is a key in the development of efficient therapeutics.

Although currently almost no data are available relating to epigenetics and arteriogenesis or collaterogenesis, it is apparent that epigenetic regulation plays a major role in both processes, and that mechanical forces such as shear stress are a determinant of epigenetic actions.

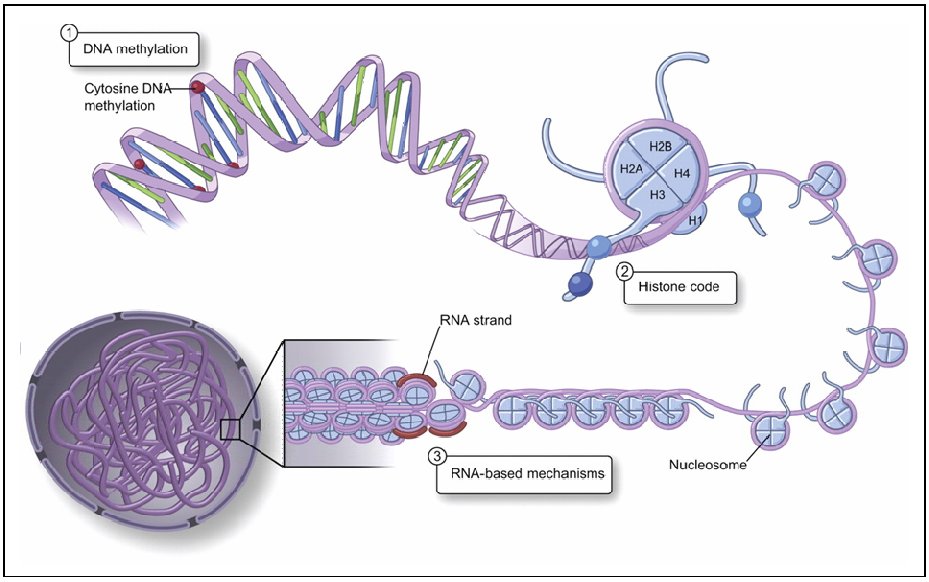

Figure 1: The figure illustrates the three basic mechanisms of epigenetic gene regulation. 1. DNA methylation, 2. histone modifications, and 3. RNA-based mechanisms (from [6], with courtesy of Journal of Applied Physiology).

A short overview of epigenetic mechanisms

The three pillars of epigenetic modifications (for a summary see Table 1) have the function to modulate the structure and accessibility of DNA, thereby controlling transcriptional activity (for an overview, see [6]: They all are involved in the formation of euchromatin and heterochromatin. Euchromatin (decondensed chromatin) is actively transcribed and affiliated with activating epigenetic marks, whereas heterochromatin (condensed chromatin) has only limited transcription and is associated with repressive epigenetic marks [7].

DNA methylation

In embryonic development, the best understood epigenetic mark is DNA methylation [8]. It involves the covalent modification of the 5-position of cytosine to define the "fifth base of DNA," 5-methyl-cytosine [2], and was already described in 1925 by Johnson and Coghill, before the elucidation of the DNA double helix [9]. The unique pyrimidine continues to base pair with guanine. In mammals, DNA methylation is almost exclusively restricted to CpG dinucleotides, commonly referred to as CpG islands [10, 11]. DNA methylation is catalyzed by 3 different DNA methyltransferases (DNMTs): DNMT1, the “maintenance” methyltransferase, which transmits DNA methylation patterns during mitotic cell division, and DMNT3a and DMNT3b, which work as de novo methyltransferases and establish DNA methylation patterns during embryonic development [10].

Histone modifications

In the nucleus, DNA is packed into chromatin as repeating units of nucleosomes, which form a "beads-on-a string" structure that can compact into higher order structures to affect gene expression. Nucleosomes are composed of 146bp DNA wrapped in histone octamers (composed of two H2a, H2b, H3, and H4) and are connected by a linker DNA, which can associate with histone H1 to form heterochromatin [10]. Histone proteins contain both a globular and an amino-terminal domain, with the latter being posttranslationally modified. Up to now, more than 60 modifications have been described, including the posttranslational modification of lysine (acetylation, methylation, ubiquitination, sumolyation), arginine (methylation) and serine and threonine (phosphorylation) [12, 13]. Many of these modifications are known to play functional roles in transcription.

The functional roles of lysine acetylation and methylation on gene expression are the best understood. Histone acetylation enhances transcription by neutralizing the basic charges of lysine residues thereby preventing chromatin compactation [14]. The impact of histone lysine methylation on gene expression, however, is dependent on the specific lysine residue. Genome-wide profiles of histone methylation show that H3K4 and H3K6 methylation are associated with transcriptionally permissive chromatin, whereas H3K9 and H3K27 methylation are markers of transcriptionally silent chromatin [15]. Furthermore, single lysine residues are variable methylated to mono-, di-, or trimethylated states. The different histone methylation states are functionally relevant. Active promoters are enriched in trimethylated H3K4, while enhancer elements are enriched in monomethylated H3K4 [16]. Similar to histone acetylation and deacetylation, which are mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), histone methylation status at a particular lysine is dynamically regulated by histone methyltransferases and histone demethylases [17].

Table 1: Summary of epigenetic control mechanisms

Mechanism	Transcriptional Effect
DNA methylation (CpG dinucleotides)	↓
Histone posttranslational modifications	
Histone H3	
Acetylation	↑
Methylation	↑/↓
Phosphorylation	↑/↓
Histone H4	
Acetylation	↑
Methylation	↓
Histone H2A	
Ubiquitination	↓
Histone H2B	
Ubiquitination	↑
RNA-based mechanisms	
si RNA	↓
ncRNA	↑/↓

The table summarizes the major known epigenetic control mechanisms on DNA, Histone and RNA level [6, 18].

RNA-based mechanisms:

RNA-based mechanisms of epigenetic regulation involve the coordinated activities of both siRNAs and noncoding (nc) RNAs, together with other epigenetic activities, such as DNA methylation and histone posttranslational modifications. A large number of intervening non-coding RNAs (lincRNAs) have been described in the genomes of human and mice, and about 3.300 lincRNAs have been identified with computationally predicted roles in various cellular processes, including cell-cycle regulation [19, 20].

DNA methylation and repressive histone modifications have been shown to be elicited at target gene promoters following treatment of cells with exogenous administration of siRNAs, and a similar phenomenon may be mediated by endogenous miRNAs [21].

Epigenetic Pathways in Vascular Biology

Non-Mendelian diseases

Common cardiovascular diseases demonstrate non-Mendelian patterns of inheritance. Nonetheless, much can be learned from such diseases, for example from Hutchon-Gilford progeria syndrome (HGPS), a childhood disease of premature aging. Affected children develop severe atherosclerosis and die from myocardial infarction and stroke at about 13 years of age [22]. HGPS results from a specific mutation (a C-to-T substitution) in the LMNA gene coding for laminin A [23]. The genetic mutation activates a cryptic splice donor site resulting in a new mRNA species that is translated to progerin. Although it is unclear how progerin causes HGPS, evidence suggests that changes in epigenetic pathways are involved. Similar to normally aged cells, the normal organization and structure of chromatin is disrupted in HGPS nuclei [23-26]. In particular, these cells demonstrate dramatically reduced heterochromatin (regions of limited transcription) that is preceded by the progressive loss of repressive epigenetic marks including trimethylated H3K9 and H3K27 [27]. In terms of collateral artery growth, it is interesting to note that progerin activates the effectors of Notch signaling pathways (see chapter 3).

Involvement of shear stress

Interestingly, laminar shear stress can elicit both global and gene-specific histone modification changes in cultured human endothelial cells (ECs) [28]. Shear stress can affect changes in global histone modification in mouse ES cells and promote their differentiation into an EC lineage [29, 30]. Laminar shear stress can also induce histone modifications at specific sites in the genome as demonstrated by the dependency on p33/HAT mediated H3 and H4 acetylation in laminar flow induced eNOS-expression [31]. Whether epigenetic pathways contribute to different regions in the vasculature in a different manner and what their functional role may be in arteriogenesis is worthwhile considering.

Endothelial cell specific gene expression

eNOS

The contribution of epigenetic pathways in endothelial response to external stimuli such as physical forces (e.g. shear stress), hypoxia, cytokines [32-34], and entry into cell cycle [18] have recently been described. Using eNOS as an example, it is evident that epigenetics provides a new vantage point for understanding transcriptional control paradigms in vascular endothelial cells (for an overview see [6]). Although eNOS can be expressed by other cell types, it is mainly transcribed by vascular endothelial cells. eNOS deficient mice are characterized by a variety of malfunctions, among them impaired wound healing, angiogenesis and reduced vascular leakage during inflammation, as well as impaired arteriogenesis ([35-37] and see chapter 6). Due to the pivotal physiological role of eNOS in the vascular endothelium, its regulation has been extensively studied.

eNOS is probably the best characterized example of an EC-specific gene that is regulated by chromatin accessibility. eNOS evidences a TATA-less promoter with two 5´cis regulatory elements, known as positive regulatory element I and II (pos. -104/-95 and -14/-155 [38, 39]. Furthermore, eNOS has a 269bp enhancer that is located -4,9kb from the transcriptional start site [40]. Similar to other EC-restricted genes, the regulatory DNA elements of eNOS can bind ubiquitous transcription factors, such as Sp-1 and Ets [18, 40]. Although transient transfection experiments of eNOS promoter-reporter constructs into various cell types showed robust promoter activity [41], eNOS promoter-reporter transgenic mice show endothelial restricted expression [42]. These data suggest the chromatin context of eNOS is involved in regulating EC-specific expression. In fact, the chromatin structure at the eNOS promoter is transcriptionally permissive in ECs and repressive in non-ECs. To date, the eNOS promoter in ECs was found to be DNA hypomethylated and enriched with activating histone post-transcriptional modifications, including acetylated H3K9, acetylated H4K12 , and di- and trimethylated H3K4 [43, 44]. In contrast, analyses of the promoter in non-expressing cells, such as vascular smooth muscle cells (vSMCs), showed DNA hypermethylation and a lack of histone posttranslational modifications. Consistent with the differences in chromatin structure of the eNOS promoter, chromatin immunoprecipitation analyses showed selected recruitment of Sp1, Sp3, Ets transcription factors and RNA polymerase II to the eNOS proximal promoter in ECs, while MeCP2 (methyl CpG binding protein 2**)** and HDAC1 were specifically localized to the promoter in in vSMCs [41, 44, 45]. Pharmacological inhibition studies demonstrated the functional importance of the eNOS promoter. While treatment of vSMCs with 5-azazytidine, a DNMT inhibitor, as well as trichostatin A, a HDAC inhibitor, resulted in upregulation of eNOS mRNA expression, treatment of ECs with methylthioadenosine, a H3K4 methylation inhibitor, provoked downregulation of eNOS expression [6].

eNOS is furthermore regulated by RNA-based mechanisms. A 27 nucleotid RNA duplex produced at the variable number tandem repeat region (VNTR) of inton 4 in eNOS was demonstrated to be expressed and localized exclusively in the nucleus of ECs [46, 47]. Experimental exogenous administration of the small 27 nt RNA to ECs induced H3K9 and H4K12 hypoacetylation at the eNOS promoter and reduced transcription [46, 48]. The repressive function of the 27 nt RNA was supported by the ability of salvage eNOS expression in the small RNA transfected cells by HDACIII depletion and treatments with trichostatin A and 5-azacytidine [48]. It is worthwhile to note that the copy number and polymorphism of the eNOS VNTR have been described to be associated with risk for ischemic heart disease [43].

In summary, chromatin-based mechanisms of gene regulation ensure that eNOS expression is mainly restricted to ECs. Furthermore, it is interesting to note that chromatin-based gene regulation is also observed in other EC-restricted genes, including vWF [49], Notch-4 [50], E-selectin [51-53] and EPHB4 [54].

iNOS

Whereas eNOS is mainly expressed by ECs, inducible nitric oxide synthase (iNOS) is silenced in the same cell type. In a recent study, Chan et al. [32] showed that the human iNOS promoter is epigenetically repressed by DNA methylation and histone H3 lysine 9 methylation. Whereas in human cell types notoriously resistant to iNOS induction (e.g., primary endothelial cells and vascular smooth muscle cells), the NOS2A proximal promoter was densely methylated at CpG dinucleotides human primary cell types and transformed cell lines capable of iNOS induction (e.g. the human colon carcinoma cell line DLD-1) had a lower density of methylated CpGs at the NOS2A proximal promoter. The prolonged inhibition of DNA methyltransferase activity in human DLD-1 cells resulted in DNA demethylation of the iNOS promoter and a 3-fold increase in cytokine-induced iNOS mRNA expression. ECs, however, were resistant to DNA demethylation, and iNOS remained silenced even after repeated 5-azacytidine treatments. These findings suggested that additional repressive epigenetic mechanisms are contributing to DNA methylation-dependent NOS2A silencing in ECs. Studies on Neurospora crassa and Arabidopsis thaliana indicated that maintenance of DNA methylation is dependent upon histone H3 lysine 9 methylation [55, 56]. And indeed histone H3 lysine 9 methylation was identified as an additional epigenetic

modification responsible for maintaining the silenced state at the NOS2A promoter in human ECs [32].

Furthermore, it was found that the iNOS promoter DNA methylation status varied strongly between human and mouse, i.e. the murine iNOS proximal promoter was hypomethylated relative to human sequences indicating that differential DNA methylation of the human and mouse iNOS promoters contributes to the marked difference in cytokine-induced gene expression [32].

Smooth muscle cell specific gene expression

Up to now, no endothelial-specific master regulator has been identified [57]. However, recently a PRDM6, a PR/SET domain containing protein was identified in flk1(+) cells at selected time points during differentiation [58]. The zinc finger protein belongs to the PRDM (*P*RDI-BF1 and *R*IZ homology *dom*ain) family of transcriptional repressors [59] named PRISM (*PR* domain *i*n *s*mooth *m*uscle), containing a modified SET domain (named for the *Drosophila melanogaster* proteins *S*u(var)3-9, *E*nhancer-of-zeste, and *T*rithorax (reviewed in reference [60]). Like other SET [61] and PR [62] proteins, PRISM are known to interact with class I HDACs [63]. PRDM6, is enriched in flk1(+) hematovascular precursor cells [58] and was also detected in embryonic and adult-derived endothelial cell lines. PRDM6 is co-localized with histone H4 and methylates H4–K20 (but not H3) *in vitro* and *in vivo*, which is consistent with the known participation of PR domains in histone methyltransferase activity. Overexpression of PRDM6 in mouse embryonic endothelial cells inhibited cell proliferation and induced apoptosis in ECs, but not in SMCs [58]. Based on the finding that PRDM6 is preferentially expressed in flk1(+) vascular precursor cells and its differential effects on endothelial and smooth muscle cells by modulating local chromatin-remodeling activity within hematovascular subpopulations during development, it was speculated that PRDM6 may coordinate cell fate decisions in vascular progenitor cells that favor smooth muscle over endothelial differentiation, or may selectively ablate cells progressing down the endothelial lineage during early stages of vascular remodeling [58].

Interestingly, a splice variant of PRDM6 was recently identified in a search for mRNAs highly expressed in aortic smooth muscle [63]. The protein was characterized as an SMC-restricted epigenetic regulator, and therefore named PRISM/PRDM6. In contrast to PRMD6, which methylates histone H4 at residue 20, PRISM/PRDM6 associates with G9a and methylates histone H3 [63]. Overexpression of PRISM in cultured primary SMCs induced expression of genes associated with the proliferative smooth muscle phenotype while repressing regulators of differentiation, including myocardin and GATA-6. Conversely, small interfering RNA-mediated knockdown of PRISM slowed cell growth and induced myocardin, GATA-6, and markers of SMC differentiation [63]. Myocardin is known to recruit histone acetyltransferase activity to the promoters of smooth muscle contractile genes [64], thereby resulting in gene activation. In addition to myocardin, GATA-6, a member of the GATA family of zinc finger transcription factors [65], promotes the contractile or differentiated smooth muscle phenotype through the induction of the cyclin-dependent kinase inhibitor p21(Cip1) [66].

However, the best-characterized regulator of smooth muscle gene expression is serum response factor (SRF), which binds a DNA sequence known as a CArG box and recruits members of the myocardin family of coactivators to activate smooth muscle contractile protein genes [67, 68]. The MADS-box transcription factor SRF plays a central role in arteriogenesis (see chapter 5 and below).

Signal transduction cascades in arteriogenesis

Growth factor signaling provokes SMCs to reenter the cell cycle with a consequent activation of genes involved in proliferation and a concomitant down-regulation of contractile transcripts [69]. Reportedly, arteriogenesis relies on the activation of the endothelium by increased shear stress. It is assumed that one or several shear stress induced endothelial mediators, in conjunction with recruitment of macrophages, promote vascular cell proliferation as a prerequisite for collateral growth and remodelling. Endothelium derived platelet derived

growth factor (PDGF) seems to be one of these mediators as its expression is differentially controlled by shear stress [70]. A recent publication demonstrates that endothelial cells derived from old animals do not express PDGF anymore, suggesting altered epigenetic control [71]. Co-administration of PDGF-BB and fibroblast growth factor-2 (FGF-2), another arteriogenic factor, has recently been described not only to promote arteriogenesis, but to induce vascular networks that remain stable for more than a year [72]. Most importantly, FGF-2 is also regulated epigenetically [73]. Furthermore, collateral artery growth is associated with an activation of the Rho-GTPase pathway (regulating actin dynamics) in smooth muscle cells, leading to altered activity of SRF that binds as a homodimer to CArG box DNA and activates transcription of genes involved in both muscle differentiation (α-SMA, SM-MHC, SM22α) and proliferation (early growth response-1, Egr-1) [71, 74, 75]. All of these smooth muscle genes have been found to be differentially expressed during arteriogenesis (own unpublished data), reflecting a switch from the contractile to the synthetic (proliferating) phenotype of vSMCs. Recently, it has been shown that vSMC-restricted binding of SRF to murine vSMC CArG box chromatin is associated with patterns of posttranslational histone modifications within this chromatin that are specific for the vSMC lineage in vitro and in vivo, including methylation and acetylation to histone H3 and H4 residues [76]. Myocardin (MYOCD), a SRF transcriptional cofactor, is essential for cardiac and smooth muscle development and differentiation. Myocardin increased SRF association with methylated histones and CArG box chromatin during activation of SMC gene transcription, whereas the myogenic repressor Kruppel like factor 4 (KLF4) recruited histone H4 deacetylase activity to vSMC genes and blocked SRF association with methylated histones and CArG box chromatin during repression of SMC gene expression. PDGF-BB treatment resulting in proliferation of vSMCs increased expression of KLF4 and decreased expression of myocardin being associated with decreases in SRF binding and H4Ac at CArG boxes [76].

Together these data indicate that epigenetic mechanisms are likely to control the expression and function of key mediators in arteriogenesis (see also chapter 5) and may account for reduced arteriogenesis observed in senescent animals (own unpublished data).

Bone marrow derived cells

A variety of studies are available describing epigenetic modification in bone marrow derived cells such as monocytes and macrophages or T-cells. In 2005, Fraga et al published a landmark report describing global and locus-specific differences in DNA methylation and histone H3 and H4 acetylation in peripheral blood lymphocytes in a large cohort of young and elderly MZ twins [77]. Of 40 twin pairs, 35% demonstrated significant differences in the three epigenetic marks. Remarkably, these epigenetically discordant twins were more likely to be older, spent less of their lifetime together, and reported the greatest differences in natural health/medical history.

Aging is known to be associated with global hypomethylation and dense hypermethylation of certain CpG islands presenting a major risk factor for atherosclerosis [78]. A global DNA hypomethylation has been observed in vascular lesions and leukocytes of atherosclerosis patients and proliferating SMCs in animal models [79-81].

An interesting study was recently published by Lund et al [82]. Investigating Apolipoprotein E deficient mice - genetically prone to atherosclerosis - they found that DNA methylation polymorphism were significantly more frequent in circulating inflammatory cells, and/or immune cells (as well as aorta) [83, 84], in comparison to control tissue (liver, pericardial fat, skeletal muscle), even before the appearance of histologically detectable vascular lesions. In particular, they found a significant global hypomethylation of genomic DNA in PBMCs (peripheral blood mononuclear cells) as well as in aorta. Furthermore, they showed for the first time that atherogenic lipoproteins promote global DNA hypermethylation in a human monocyte cell line, i.e. THP-1 cells. In this current volume, Vincent van Weel (Chapter 9) reports on the role of bone marrow derived cells in arteriogenesis. He previously published a study showing that perfusion recovery was strongly decreased in APOE3*Leiden mice after femoral artery occlusion, as well as that hypercholesterolemia is associated with reduced arteriogenesis [85].

Is there a need for stem cells?

Epigenetic research is of fundamental relevance for stem cell technology. Stem cells are able to differentiate into all cells of a body. Stem cells, as well as each cell of the body, contain the same DNA sequence and genes. Epigenetic modifications, however, control gene usage. Stem cells are able to give rise to every cell type and studies indicate that their DNA is more open to instructions and "manipulations", i.e. their epigenetic instructions are missing and not yet enforced [3]. Reprogramming differentiated cells in terms of epigenetic remodifications may present one of the utmost future challenges.

Conclusion

Epigenetics is a relatively new field in research. Epigenetic modifications present a check point of gene expression affecting each cell of a body. They are upstream of transcription activators or –repressors and therefore have to be recognized at a more global level. Although currently no data of direct investigations on epigenetic modifications in arteriogenesis or collaterogenesis are available, it is clearly evident that they must play a major role in both processes.

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Chapter 8. Thymosin ß4 a Promising Therapeutic Agent to Promote Arteriogenesis

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Abstract: Thymosin ß4 is a small water soluble peptide of 5 kDa, which was isolated by Goldstein and White in 1966 from the thymic gland [1]. Since then, its clear role in distinct processes such as wound healing [2], hair growth via stem cell migration [3,4], cardioprotection [5] and angiogenesis [6] is contrasted by a high level of uncertainty with respect to the exact mechanisms providing these pleiotropic effects (Table 1). However, several approaches render thymosin ß4 as a possible candidate for future clinical therapy, including cardioprotection and neovascularisation. Therefore, we focus on the modes of action involved in thymosin ß4 signaling in the cardiovascular system, in particular its significance for angiogenesis and arteriogenesis.

Table 1 Pleiotropic effects of Tß4

Function	References
Accelerates wound healing	[2]
Hair follicle growth via activation of stem cell migration	[3 ,4]
Sequesters actin	[10, 11]
Enhances angiogenesis	[17, 18]
Necessary for embryonic coronary vessel formation	[27]
Cardioprotective following myocardial infarction	[30]
Downregulates inflammatory cytokines and chemokines	[53, 54]
Promotes corneal wound healing	[53, 54, 55]
Supresses NF-κb after TNF-α stimulation	[55]

Family and background:

In the 1960s the laboratory of Abraham White at the Albert Einstein College of Medicine in New York firstly isolated and partially purified a group of hormon-like peptides from the thymic gland and coined the term thymosins for them [1]. Although initially thought to be a single polypetide of approximately 14 kDa thymosins later turned out to be a mixture of several peptides weighing 1 to 15 kDa. Due to 5 steps of preparation, this mixture was named thymosin fraction 5 [7,8]. This family of thymosins consisting of a number of small peptides received their nomenclature from their isoelectric points: α- thymosins below a pH of 5.0, ß-thymosins between a pH of 5.0 – 7.0 and γ-thymosins with a pH of 7.0 and higher [9].

Two members of this thymosin fraction 5 family, thymosin α1 and ß4, are in advanced stages of clinical trials. Here we want to focus exclusively on the functions and interactions of thymosin ß4.

Thymosin ß4 and actin

In 1990 Dan Saver and his colleagues were able to show that thymosin ß4 plays an important role in the sequestration of actin [10,11]. Actin is the major molecule of cellular locomotion, cell shape, cell division and intracellular transport. These dynamic changes of the cytoskeleton require rapid assembly of monomeric g-actin to filamentous f-actin and in return quick disassembly. Looking at purified actin under ionic conditions in a test tube, more than 90% would polymerise forming f-actin while resting cells show equal amounts of f- and g-actin [12]. How does the cell manage to keep this constant pool of single actin molecules? Specific actin-monomer binding proteins are indispensible. The most abundant protein complexing with g-actin and preventing its polymerisation is thymosin ß4, which can be found in almost any eukaryotic cell, binding monomeric g-actin in a stoichometric 1:1 complex preventing it from ionic induced assembly. Stimulation of cells induces actin polymerisation via filament-barbed-ends. In this case free monomeric actin is provided either by dissolving of the thymosin- actin complex or by aggregation of g-actin with profilin. Profilin bound actin may polymerise forming changes in cellular shape. To complete the cycle of actin organization, f-actin is depolymerised through cofilin and gesolin family proteins resulting in monomeric g-actin (Fig. **1**) [13,14]. Tight regulation of these binding proteins is mediated by changes in intracellular calcium-ion concentration, phosphorlylation of branching sites and various pathways [15].

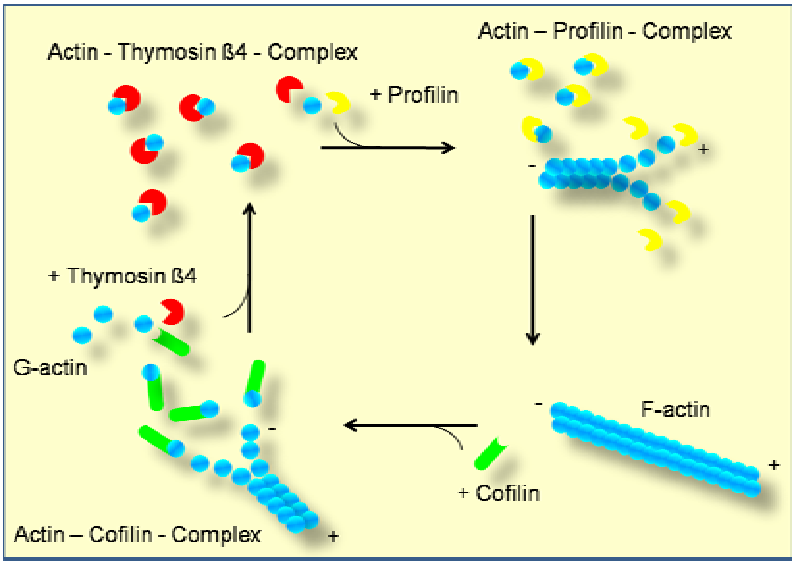

Figure 1: Thymosin ß4 sequesters monomeric g-actin, while profilin assists in f-actin polymerisation. Cofilin is involved in the process of depolymerisation from f-actin to g-actin.

Thymosin ß4 and angiogenesis

The first hint of thymosin ß4 mediating angiogenesis was observed in Matrigel assays by Kleinman and Goldstein in 1995 [16]. Endothelial cells transfected with thymosin ß4 started to spread and attach significantly higher on Matrigel than control cells and rapidly arranged into capillary-like tube networks. I*n vitro* thymosin ß4 acts as a chemoattractant for endothelial cells. In vivo, using the subcutaneous implantation of Matrigel thymosin ß4 forced endothelial cell migration [17]. Vascular sprouting of coronary artery rings accelerated after exogenous application of only 100 ng thymosin ß4 resulting in a doubling of the vessel area [18]. Interestingly, uptake of thymosin ß4 via internalization or binding to an unknown cell surface receptor was suggested, implying both an autocrine and paracrine role of thymosin ß4

in vessel formation. Due to the fact that controlled angiogenesis is essential for effective wound healing, thymosin ß4 increased wound healing rates, collagen deposition and keratinocyte migration [2].

Role of a cleavage product of thymosin ß4: AcSDKP

Besides the pleiotropic roles of full-length thymosin ß4, cleavage of its N-terminus between the amino acids Pro 4 and Asp 5 by an endopeptidase [19] generates a bioactive fragment namely Ac-SDKP. Ac-SDKP is ubiquitously present in various tissues and occurs in the plasma at nanomolar concentrations [20]. The angiotensin- converting- enzyme (ACE) is responsible for Ac-SDKP degradation and ACE-inhibitor therapy results in increased plasma levels of Ac-SDKP [21]. This might contribute to the effects seen after ACE-Inhibitor-administration in patients. Ac-SDKP has been shown to provide angiogenesis in vitro and in vivo [22] and suffices to enhance neovascularisation in hindlimb ischemia through MCP-1 signaling [23]. MCP-1 induces upregulation of hypoxia-inducible-factor-1-alpha (HIF-1α) and consequently vascular endothelial growth factor-A (VEGF-A) expression [24]. Additionally, MCP-1 attracts monocytes to the area of conductance vessel maturation as an important part of arteriogenesis [25]. Concurrent with these findings systemic administration of Ac-SDKP accelerated revascularization following myocardial infarction [26] and knockdown of thymosin ß4 in the developing heart was accompanied by a significant reduction in Ac-SDKP level [27]. Attempts to restore full function in the embryonic cardiac setting lacking thymosin ß4 by injecting Ac-SDKP into pregnant female mice failed, demonstrating the importance of the full-length protein at least during embryonic vasculogenesis. However, in adult epicardial explants Ac-SDKP induced differentiation of Flk-1^{+} progenitor cells to endothelial cells but not smooth muscle cells, unlike Tß4 administration. Furthermore, Ac-SDKP was not sufficient to induce a significant outgrowth [28]. Interestingly, Ac-SDKP provides anti-fibrotic qualities eventually contributing to its cardioprotective effect as after myocardial infarction Ac-SDKP lowers the remodeling from necrosis to fibrosis resulting in decreased scar formation [29].

Cardioprotective effect of Thymosin ß4 during myocardial ischemia – reperfusion

Since repair mechanisms are predominant in wound healing after coronary occlusion, several laboratories followed the hypothesis that thymosin ß4 would contribute to the limitation of tissue damage and adverse remodeling after myocardial ischemia. Ildiko Bock- Marquette and Deepak Srivastava first unmasked the potential of thymosin ß4 to increase post-ischemic cardiomyocyte survival in a murine model of myocardial infarction [30]. Surprisingly, this pro-survival capacity still held up when thymosin ß4 was applied after the initiation of ischemia, a scenario most likely occuring under clinical circumstances.

Supporting this notion, we used a preclinical model of ischemia and reperfusion in pigs for the assessment of acute cardioprotection. We observed an enhanced myocyte survival at 24h after reopening the occluded LAD upon regional thymosin ß4 application into the ischemic area [5], involving a decrease in apoptosis and postischemic inflammation.

Intracellular modes of action of Thymosin ß4

Regarding the mode of action of thymosin ß4 signaling, several possibilities exist. Despite intense research, no receptor structure has been revealed yet (Fig. **2**), rendering other possibilities likely. Bock-Marquette demonstrated intracellular thymosin ß4 uptake using phage particles as a carrier. Phage particle expressing thymosin ß4 on their surface were added to mouse embryonic heart explants and then detected using an antiphage antibody. The antiphage antibody covered the cell surface, parts of the cytosol and was even detectable in the nucleus whereas control phages without thymosin ß4 expression were not traceable throughout the cells [31]. Similarly, Huff et al. demonstrated a nuclear uptake of exogenously applied thymosin ß4, postulating an active uptake mechanism [32]. Exogenous administration of thymosin ß4 onto a system of embryonic heart explants promoted cardiomyocyte and endothelial cell migration away from the explant. Cardiac myocytes which usually do not

migrate were also stimulated in migration identified as spontaneously beating, cardiac muscle -actin positive cells. Besides neonatal cardiomyocytes treated with thymosin ß4 showed an increase in survival, they were able to contract regularly and for a longer period of time than controls [31].

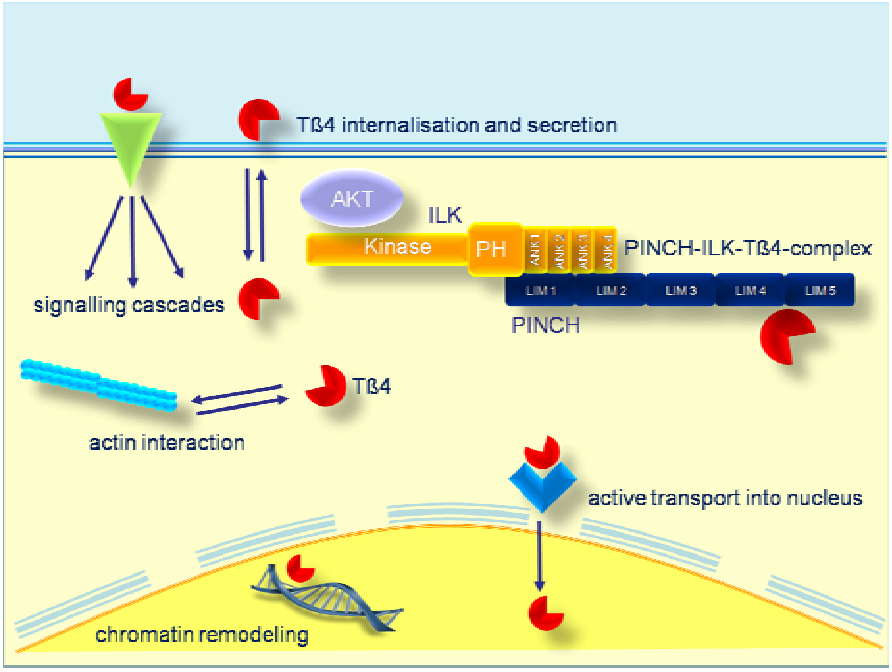

Figure. 2: Suggested mechanisms of thymosin ß4 signalling: Thymosin ß4 is proposed to enter the cell via internalisation or providing its effects via a cell surface receptor. Conversely it is likely to be secreted. Intracellulary it regulates actin sequestration and can be found as part of a ternary complex with PINCH and ILK activating the AKT. Thymosin ß4 can also enter the nucleus, maybe using an active transport mechanism and mediate gene transription.

The exact mechanism of action how thymosin ß4 is able to promote migration and survival is unclear but at least some interactions are well described. It has been reported recently that thymosin ß4 interacts with PINCH (Particularly Interesting New Cys-His Protein) a LIM domain protein. PINCH interacts through its first LIM domain with the Integrin–Linked-Kinase (ILK) building a complex which indirectly takes part in the regulation of the actin cytoskeleton assisting in connecting to the extracellular matrix. This expanded complex is also known as the focal adhesion complex, being centrally involved in cell motility [33], cell modulation and cell survival and optimal activation of the Proteinkinase-B (AKT) [34]. Phosphorylation of the serin-threoin-kinase AKT is a central step of signaling pathways involved in cell survival and growth [35,36]. Thymosin ß4 binds PINCH and ILK independently showing a stronger interaction with PINCH, being integrated into this ternary complex of all three proteins (Fig. **2**). In comparison to the ILK interacting with the first LIM domain of PINCH, thymosin ß4 connects to the complex at the fourth and fifth out of five LIM domains of PINCH. In addition to thymosin ß4 operating with ILK and PINCH, treatment of C2C12 cells with thymosin ß4 showed increased levels of ILK and its substrate P-AKT [27]. Activation of the AKT requires phosphorylation of two amino acids, Thr 308 and the Ser 473. Thr 308 is phosphorylated by the enzyme PDK1 whereas the kinase for Ser 473 phosphorylation has not been identified yet [37] but a possible candidate could be the ILK. Consistent with this hypothesis the therapy of infarcted mice hearts with thymosin ß4

accelerated levels of ILK and Ser 473 phosphorylation of the AKT [30]. These results demonstrate a possible pathway resulting in activation of the AKT/ protein kinase B consistent with the recent finding of thymosin ß4-induced protein kinase C activation [38], a different pathway of AKT activation.

Thymosin ß4 is essential for coronary vessel formation

Thymosin ß4 is known to be upregulated in early embryogenesis of mice, in particular in regions of blood vessel formation and in endocardial cushions [39]. The conventional formation of coronary blood vessels requires growth and maturation of microcirculatory as well as macrocirculatory vessel. Smart et al. identified thymosin ß4 as essential for all three steps in the developing heart. During coronary vessel formation in embryonic mice epicardial progenitor cells differentiate and migrate into the inner layers of the developing heart giving rise to a vascular network sufficient to feed the growing myocard [40,41,42]. Transgenic mice carrying an alpha-MHC-restricted knock-down of thymosin ß4 are still able to induce transformation of these epicardial derived cells but lack migration into the inner layers. Subsequent to missing migration, thymosin ß4 knockdown hearts display clusters of immobilized endothelial and smooth muscle cells in the epicardial layer. Interestingly, besides coronary vessel formation the development of large thoracic vessels, e.g. branching of the aorta and subclavian arteries was impaired due to a lack of smooth muscle cell migration [27]. Recent data also identified thymosin ß4 as a stimulator of actin-dependant pseudopodia formations which are of utmost importance for cell migration as they determine the direction of movement [43].These findings suggest a paracrine role of thymosin ß4 signaling between the myocard and the epicard as a key step in embryonic cardiac vessel formation [44].

eEPCs, thymosin ß4 and arteriogenesis

The contribution of endothelial progenitor cells (EPC) to new blood vessel formation is a central subject for current investigations. Recent evidence supports the concept of progenitor cells contributing to neovascularization via secretion of paracrine factors. Earlier work of our lab demonstrated the beneficial role of eEPCs [45] in two animal models of acute and chronic ischemia. The local retroinfusion of eEPCs into the ischemic hindlimb of rabbits as well as the systemic injection following myocardial infarction in mice showed an increase in neovascularisation and promotion of tissue recovery [46]. Embryonic EPCs increased limb perfusion via capillary sprouting and conductance vessel formation in rabbits and limited cardiac dysfunction in mice. Additionally, eEPCs reduced ischemia reperfusion injury in pigs involving the PI3/AKT pathway [47].

To further analyze the contribution of eEPCs to cardiomyocyte survival and vessel formation, we performed a transcriptome analysis of eEPCs, revealing a broad range of angiogenetic factors, among others thymosin ß4. Indeed, the cardioprotection provided by embryonic EPCs was lost when thymosin ß4 was knocked down using a thymosin ß4 specific shRNA [5]. In vitro cardiomyocytes exposed to hypoxia and reoxygenation improved in survival after eEPC treatment unless thymosin ß4 was knocked down using shRNA. Similar endothelial cell apoptosis was reduced in a setting of eEPC treatment apart from shRNA transfection. Consistently, a lack of Tß4 in eEPCs abrogated their cardioprotective potential, whereas Tß4 alone sufficed to limit infarct size and postischemic inflammation [48].

As mentioned earlier the model of chronic hindlimb ischemia in rabbits showed beneficial effects on angiogenesis and arteriogenesis after treatment with eEPCs. The knock down of Tß4 in eEPCs blunted the neovasculatory response usually obtained after application of wildtype eEPCs into the chronic ischemic limb vasculature. Of note, conductance vessels (Fig. **3A**, Fig. **4**) were affected to a similar extent as microcirculatory vessels (Fig. **3B**), diminishing the resulting blood flow (Fig. **3C**). Thus, although eEPCs secrete a variety of proteins involved in the process of neovascularisation, thymosin ß4 expression appears indispensable for their neovasculatory response in vivo [49].

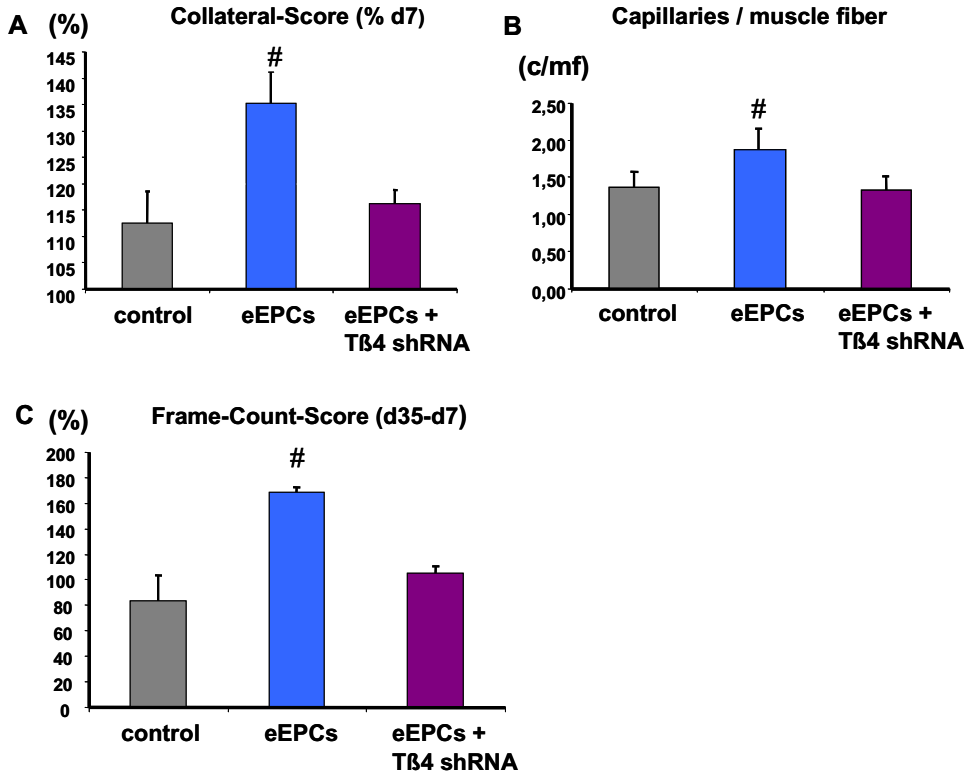

Figure 3: Postischemic neovascularisation in the hindlimb of rabbits. A & C, Collateral growth and perfusion were increased after regional retroinfusion of eEPCs , unless Tß4 was down regulated. B, Quantification of capillaries per muscle fiber of the ischemic calf muscles demonstrating accleration of capillaries after eEPC application, whereas sh-RNA cotransfection blunted these effects. (n=3; * P< 0,05 vs. control)

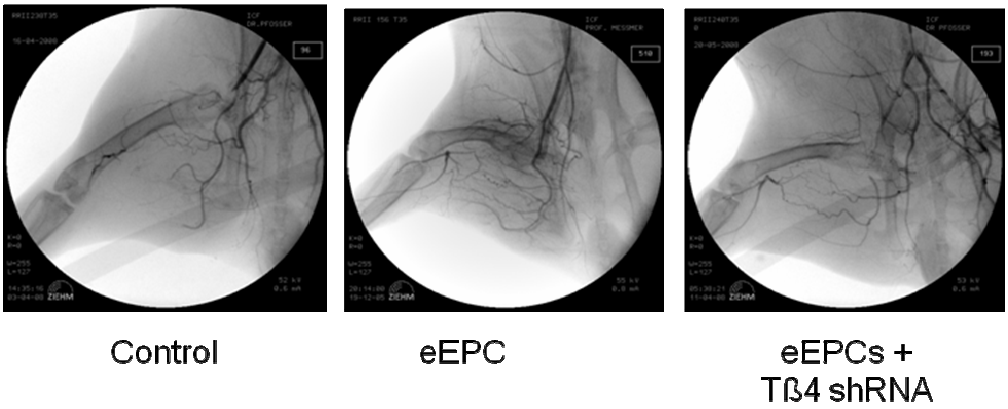

Figure 4: Angiographies after 35 days in a model of chronic hindlimb ischemia in rabbits. The treatment with eEPCs showed an increase in collateral formation in comparison to the control group, whereas co-application of thymosin ß4 shRNA abolished this effect [49].

Our findings reveal a surprising effect: thymosin ß4, a small protein might be the key factor in eEPC mediated stimulation of arteriogenesis. It will be interesting to see, if this protein is capable of stimulating arteriogenesis and showing anti-inflammatory properties (see below) at the same time, a combination which is of utmost importance for a future therapeutic use.

Thymosin ß4 in inflammation:

Atherosclerosis is a disease caused by chronic endothelial activation and monocyte/macrophage recruitment during the process of plaque formation [50,49]. On the other hand, recruitment of monocytes is a central function of arteriogenesis [50], which at times was experimentally enhanced by pro-inflammatory stimuli. Given the pro-arteriogenic effect of thymosin ß4, its role in defined models of inflammation was investigated by several groups.

In particular, Sosne et al analyzed a model of corneal wound healing. The local application of thymosin ß4 intensified the re-epithelialisation following corneal injury and modulated several cytokines for example interleukin (IL)-1ß and IL-18 [51]. Following alkali injury thymosin ß4 stimulated corneal wound healing and again showed anti-inflammatory properties by downregulating chemokines like macrophage inflammatory protein (MIP)-1α, MIP-1ß, MIP-2 and again the cytokine IL-1ß [52]. Using TNF-α, a well-know pro-inflammatory cytokine thymosin ß4 co-application was efficient to downregulate NF-κb activation in human corneal epithelial cells. The effect on NF-κb down regulation comprised NF-κb phosphorylation, activity and nuclear translocation [53]. Thymosin ß4 also decreased the number of inflammatory cells in corneal wound healing [52] and showed a modulation of the MMP/TIMP balance in mice after corneal alkali injury [54] and reduced the levels inflammatory chemokines and cytokines in studies of septic shock [55].

Discussion:

Thymosin ß4 contributes to a wide range of cellular processes, and a number of gene expression changes [56,57] have been reported. Infection of lung tumor cells either with an adenovirus expressing thymosin ß4 or the single peptide showed an increase in VEGF expression [58]. Additionally, thymosin ß4 knockdown hearts appeared to have lower expression levels of VEGF [27] but still it is not clear if there is a direct interaction of thymosin ß4 and VEGF.

Regarding the role of thymosin ß4 in the cytoplasm, a signaling cascade of thymosin ß4 interacting with PINCH and ILK leading to AKT activation has been shown to be relevant. But do these complex interactions require the g-actin sequestering function of thymosin ß4? Neither stimulation of f-actin formation nor the inhibition of actin polymerisation influenced the levels of ILK after thymosin ß4 administration in C2C12 cells. In addition blocking PI3-kinase, an upstream enzyme of the ILK abolished thymosin ß4 induced effects [30] showing the importance of this signaling pathway for thymosin ß4 action.

To date, options for therapeutic neovascularisation are still confined but expandable. Considering its multifarious effects on the cardiovascular system, thymosin ß4 is likely to be an auspicious therapeutic target. For instance, it is essential for embryonic coronary vessel formation [27], angiogenesis [18] and eEPC mediated arteriogenesis. Another advantageous property of thymosin ß4 is its anti-inflammatory potential [53].

Taken together thymosin ß4 is involved in any process of cellular shape and migration through its function in actin polymerization. It is of great interest to define the mechanism of action and the molecular pathways involved in thymosin ß4 signaling in health and disease. Despite a lack of mechanistically certainty, the current evidence suggests that thymosin ß4 is likely to show great promise in future therapeutic strategies in injured tissues.

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Chapter 9. Bone Marrow Derived Cells in Arteriogenesis: a Crucial Role for Leukocytes

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Abstract: In recent years cell therapeutic approaches for induction of arteriogenesis, including injection of autologous bone marrow, have gained popularity for experimental treatment of patients with coronary or peripheral arterial ischemic disease. However, beneficial results of these cell therapies in patients remain inconsistent to date. Evidence points towards a supportive paracrine role of bone marrow derived cells by secretion of various pro-arteriogenic cytokines and growth factors. Moreover, inflammatory cells, originating from the bone marrow, are recently pointed out as the key regulators of arteriogenesis. This chapter focuses on the role of leukocytes in arteriogenesis. In particular, the role of monocytes, natural killer cells and T-cells, including various subpopulations of T-cells, are addressed. Activation (or inhibition) of selective leukocyte subpopulations may ameliorate pro-arteriogenic cell therapy in the future.

Introduction

In the past ten years, therapeutic arteriogenesis using bone marrow derived cells has become a promising treatment for patients with either coronary or peripheral ischemic disease [1-6]. Although initial reports of autologous bone marrow transplantation for patients with limb ischemia were very promising [7], beneficial results of cell therapy in patients with peripheral arterial disease (PAD) are not very consistent to date [8].

Although the concept of the development of adult collateral arteries from a pre-existing arteriole network is indisputable for therapeutic intervention, the exact cellular mechanism underlying arteriogenesis remains unclear. There are different hypotheses on the role of bone marrow derived cells in arteriogenesis. Nowadays, evidence points towards a more supportive paracrine role of bone marrow derived cells by secretion of various factors instead of the initially proposed role in promoting arteriogenesis by incorporating into the vessel walls [9]. Also the role of different inflammatory cells, originating from the bone marrow, has extensively been studied in order to evolve a more efficient cell therapeutic approach than infusion of the total bone marrow derived cell fraction. Previously, numerous animal studies have established that ischemia induced angiogenesis and shear stress induced arteriogenesis can be enhanced with different types of leukocytes, cytokines and growth factors. The molecular and cellular mechanisms that play a role in collateral artery formation start to become unravelled more recently. Increased shear stress induces arteriogenesis by stimulating the attraction, adhesion and invasion of circulating inflammatory cells [10] such as monocytes, T-lymphocytes and NK-cells. Growth factors that are already present in the ischemic tissue, as well as those that are produced by the invading cells play an important role too. Recently, also subpopulations of T-lymphocytes, such as CD4+ T cells, CD8+ T cells and CD4+CD25+FoxP3+ T cells and their role in arteriogenesis have been studied in more detail. This chapter will focus on the role of leukocytes in collateral artery formation.

Monocytes in arteriogenesis

Monocytes play a crucial role in mediating neovascularization in response to increased shear stress and ischemia. Already in the 1970s monocytes were found to invade the vessel wall of developing coronary collateral arteries [11,12]. Later on, functional proof for the participation of monocytes in arteriogenesis has been reported by the results that after increasing blood monocyte concentration in a hind limb ischemia mouse model, arteriogenesis is enhanced [11-13]. In contrast, after depletion of monocytes, collateral artery formation is much

attenuated [12]. Increased shear stress, due to the arterial occlusion, activates the vascular endothelium. The activated endothelial cells upregulate the monocyte chemoattractant MCP-1 and adhesion molecules (eg. ICAM-1 and VCAM-1) to which the receptor of monocytes binds [14,15]. This leads to monocyte adhesion and infiltration with the subsequent production of growth factors and proteases. In the next paragraph we will discuss these aspects of monocytes in arteriogenesis into more detail.

The local production of cytokines and chemokines within the first 3 days after ischemia mediates the recruitment of monocytes to the ischemic area [16-19]. To stimulate arteriogenesis, monocytes must home and retain around the arterial occlusion. There is evidence that MCP-1, but also VEGF and SDF-1α contribute to the recruitment and retention of monocytes [19-23]. For example, local infusion of MCP-1 [24] or MCP-1 gene transfer [25] in a hind limb ischemia rabbit model increase the local number of monocytes in the ischemic area and enhance revascularization. Voskuil et al. [26] reported impaired monocyte recruitment after hind limb ischemia in MCP-1-/- mice. This effect was reversed with treatment of the MCP-1 protein. Furthermore, Heil et al. [27] showed that disruption of the MCP-1 receptor (CCR2) in CCR2-/- mice leads to an impaired blood flow restoration after femoral artery ligation. This impairment was based on a reduced migration capacity of these monocytes.

The interaction of the monocyte with the activated endothelium is a complex multistep process. Auffray et al. [28] reported recently the importance of the role of monocytes in the initial recruitment of blood monocytes. They observed a subset of monocytes that could crawl on endothelial cells. These resident monocytes patrol healthy blood vessels and allow rapid tissue invasion by monocytes in case of ischemic injury or tissue damage [28]. Two integrins are responsible for the interaction of monocytes with endothelial cells: Mac-1 and LFA. These intergrins interact with adhesion molecules on the endothelial surface, like selectins, intercellular adhesion molecules (ICAM-1 and ICAM-2) and vascular adhesion molecules (VCAM-1) [14]. It was demonstrated that factors like MCP-1 and VEGF, released by the activated endothelial cells, can increase the expression of integrins on monocytes and enhance monocyte adhesion [29-31]. After monocyte adhesion, integrins also mediate transmigration of monocytes into the perivascular tissue. Hoefer et al. showed that in vivo treatment with antibodies against ICAM-1 blocked collateral artery growth [32].

For invading into deeper vessel wall layers, monocytes can use their capacity to produce proteases such as matrix-metalloproteinases and u-PA. Their proteolytic activity can create gaps by which monocytes can migrate further into the vessel wall [33-35]. After transmigration into the perivascular tissue, monocytes differentiate into macrophages and secrete growth factors and cytokines that attract other inflammatory cells and stimulate smooth muscle cell proliferation and endothelial cell mitosis, both necessary for collateral growth [36]. One of the pro-inflammatory factors secreted by monocytes and macrophages is TNF-α. In a rabbit hind limb ischemia model, it is demonstrated that arteriogenesis is attenuated after treatment with TNF-α inhibitors, most likely due to the inhibition of leukocyte infiltration around collateral arteries and lower vascular smooth muscle cell proliferation [37]. Also fibroblast growth factors (FGF), provided by monocytes in a paracrine way, do have a stimulating effect on arteriogenesis [13].

The potential of transplantation of monocytes to stimulate collateral artery formation in the clinical situation has now been studied by using the hind limb ischemia animal model. Herold et al. [15] showed that although autologous monocytes only demonstrated a marginal increase in arteriogenesis, their transduction with GM-CSF before transfusion into the hind limb ischemia model resulted in a robust stimulation of arteriogenesis. Also Urbich et al. [38] provided evidence that transplantation of engineered monocytes represents a highly effective therapeutic approach to stimulate collateral artery formation. In contrast, monocytes injected directly after isolation, without any stimulation, did not show any stimulating effect on arteriogenesis. Isolation of monocytes can easily be done in humans by leukapheresis and these cells have been safely administered for patients with malignancies. The strategy of transplantation of pro-arteriogenic monocytes seems feasible, but further research towards the side effects (e.g. pro-atherogenic effects) is warranted.

T cells in arteriogenesis

More recently, it was found that other leukocytes than monocytes also contribute to collateral artery formation. T lymphocytes also play a role in many vascular diseases ranging from a pro-atherogenic role in atherosclerosis towards a beneficial role in arteriogenesis. Couffinhal [39] showed that nude mice, which are deficient for all types of mature T lymphocytes, did have a hampered capacity to form collateral arteries after induction of hind limb ischemia. The role of T lymphocytes in arteriogenesis has recently been studied more extensively, with focus on arteriogenic function of specific subpopulations of T lymphocytes. Although the exact role is not completely understood yet, we will discuss the contribution of CD4+ T cells (Helper T cells), CD8+ T cells (Cytotoxic T cells), CD3+CD31+CXCR4+ T cells (angiogenic T cells) and CD4+CD25+FoxP3+ T cells (regulatory T cells). See Figure **1** for a schematic representation.

The importance of CD4+ T cells in collateral artery formation has been demonstrated recently. CD4-/- mice, which do have a normal development of CD8+ T cells and myeloid cells showed a markedly reduction in ischemia-induced collateral artery formation. Compared to controls, there was a 25% decrease in blood flow restoration after femoral artery ligation during 28 days in these CD4-/- mice [40]. In a rescue experiment, CD4-/- mice received spleen-derived purified CD4+ T cells and restored blood flow recovery after ischemia to the levels of wild type mice [40]. Additionally, CD4 depleted mice, show significantly impaired blood flow restoration after femoral artery ligation [41]. Furthermore, MHC class II deficient mice, which are characterized by the selective lack of the maturation of CD4+ T cells, display impaired arteriogenesis too [41].

The most likely mechanisms contributing to the arteriogenic-enhancing effects of CD4+ T cells appears to be that CD4+ T cells play an important role in the classic immune response in the ischemic area. In wildtype C57BL/6 mice, CD4+ T cells accumulate around collateral arteries within 3 to 7 days after ligation of the femoral artery [41]. It is believed that perivascular presence of T cells is necessary to destroy cells to create space for collateral growth [42]. Also exogenous CD4+ T cells home to the inflammatory cell infiltration in the ischemic hind limb within 24 hours after transfusion. In contrast, CD4 negative cells do not have selective homing to the ischemic hind limb [40]. CD4+ T cells secrete various cytokines for example to induce monocytes/macrophage accumulation in the ischemic muscle [43]. These monocytes and macrophages secrete large arrays of cytokines and growth factors which further facilitate arteriogenesis [43]. Recently, van Beem et al. [44] described the role of CD4+ T cells in the stimulation of CD14+ monocytes to differentiate into a pro-angiogenic cell type, namely endothelial progenitor cell colonies (CFU-ECs). Paracrine factors produced by activated CD4+ T cells present in the ischemic tissue facilitate CFU-EC formation. The exact cocktail of soluble factors stimulating CD14+ monocytes and the role of these CD4+ T cell - stimulated monocytes need to be further investigated.

The role of CD8+ T cells in arteriogenesis is demonstrated by an impaired blood flow restoration in CD8-/- mice after hind limb ischemia. An attenuated blood flow persisted from day 3 till day 28 after femoral artery ligation compared to controls. After transfusion of exogenous CD8+ T cells into CD8-/- mice, the impaired blood flow recovery after ligation was rescued [45]. CD8+ T cells are one of the first cells involved in collateral artery formation [45]. When CD8+ T cells infiltrate into the ischemic muscle, they express IL-16, which is an important chemo-attractant for several immune cells such as monocytes, eosinophils and dendritic cells [46-48]. Similarly, IL-16 participate in the recruitment of CD4+ T cells since it has been shown that IL-16 is a natural ligand for CD4 [48].

More recently, the role of a specific subset of CD4+ T cells, the regulatory T cells, has been studied. CD4+CD25+FoxP3+ regulatory T cells constitute 10% of CD4+ T cells in the peripheral blood. This T cell population is specialized to suppress immune responses and contributes to the maintenance of immunological self-tolerance and immune homeostasis. In addition, these regulatory T cells do have an important function in the T cell homeostasis and suppress T cell responses against self-antigens or foreign antigens. Interestingly, it has been shown that regulatory T cells participate in the control of the development of atherosclerotic lesions. The role of regulatory T cells in ischemia-induced collateral artery formation has been recently studied by Zouggari et al. [49]. They modulated regulatory T cell levels by intervening in the B7/CD28 interaction, which is required for regulatory T cell generation and

homeostasis. In this regard, hind limb ischemia was induced in CD28-/- and B7-1/2-/- mice, which are deficient for regulatory T cells. In these mice, a 1.2 to 2.0 fold increase in neovascularization after hind limb ischemia induction was found. In line with this, collateral artery formation was also increased in anti-CD25-treated mice compared to controls [49]. Taken together, regulatory T cell reduction with B7- or CD28 deficiency or anti-CD25 treatment, increases post-ischemic neovascularization. So, regulatory T cells play an important role in arteriogenesis, most probably by controlling the effector immune cell response [49]. Modulation of the regulatory component of post-ischemic inflammatory cell response, could provide a novel level of therapeutic intervention for pro-arteriogenic strategies for the treatment of PAD.

Another subpopulation of T cells, CD3+CD31+CXCR4+ T cells, has been suggested as a potential target for ischemic cardiovascular diseases. This cell is also referred as angiogenic T cell in literature. Hur et al. [50] report that the central cluster of endothelial progenitor cells (EPCs), which play an important role in neovascularization [51,52], is composed of CD3+CD31+CXCR4+ T cells. These angiogenic T cells are required for colony formation and early EPC differentiation as was investigated by depletion and adding these T cells during EPC-culture.

Furthermore, CD3+CD31+CXCR4+ T cells secrete various pro-angiogenic cytokines such as VEGF, IL-8, IL-17 and granulocyte colony-stimulating factor. Moreover, these cells also secrete MMP-9 which is known to play an important role in angiogenesis related extracellular matrix degradation. In vitro experiments as tube formation assays, as well as in vivo experiments further illustrate the pro-angiogenic capacity of CD3+CD31+CXCR4+ T cells. Infusion of CD3+CD31+CXCR4+ T cells in a hind limb ischemia mouse model improved blood flow recovery significantly. Clinical studies showed that the level of angiogenic T cells in peripheral blood MNCs is decreased when risk scores for cardiovascular disease are increased. This stresses the clinical relevance of this subpopulation T cells and further research towards this cell as target for cell therapy or as biomarker of cardiovascular disease is necessary [50].

Natural killer cells in arteriogenesis

Natural Killer (NK) cells were recently proven to contribute to arteriogenesis as well [41]. As explained previously, collateral growth is initiated by shear stress induced release of chemoattractants, such as MCP-1. Not only is MCP-1 a potent chemoattractant for monocytes [24,53] but also for NK cells [54].

The role of NK cells in processes of vascular remodeling, e.g. atherosclerosis [55] but also remodeling of spiral arteries in the placenta [56] was already described. Mice deficient for functional NK cells have reduced atherosclerotic lesions and mice deficient for uterine NK (uNK) fail to modulate spiral arteries feeding the placenta.

The role for NK cells in arteriogenesis was established when these cells were found to accumulate around collateral arteries and by the observation of impaired arteriogenesis following femoral artery ligation in mice depleted or deficient for NK cells [41]. Unlike a well established role for NK-T cells in atherosclerosis [57], this could not be proven for type 1 NK-T cells in arteriogenesis. Especially since J-alpha281-knockout mice, that selectively lack V-alpha14 NK-T cells, did not show impaired arteriogenesis after femoral artery ligation [41]. However, the role of type 2 NKT cells is unclear.

NK cells are cytolytic effector cells of the innate immune system primarily involved in the defense against infectious pathogens, especially viruses. Their state of action is determined by dual signaling via inhibitory and activating surface receptors [58]. Many of these receptors, e.g. NKG2D, NKrp1c (NK1.1), CD94/NKG2, and the highly polymorphic Ly49 receptor family, code for in the Natural Killer gene complex (NKC) [58,59]. Ly49 and CD94/NKG2 receptors are expressed in a stochastic and independent fashion, which results in NK cell subsets expressing distinct combinations of these receptors. NK cell function is, in part, based on the interaction of their inhibitory receptors with Major Histocompatibility Complex class I (MHC I) molecules, expressed on the target cell surface in a complex with β2-microglobulin

(β2m) [60]. Often, activating NK cell receptors recognize inducible molecules on the target cell, which can be induced upon cellular stress such as hypoxia or infection [61].

Upon activation NK cells produce a variety of cytokines, e.g. perforin, TNF-α, TNF-β, IL10 and most dominantly IFN-γ and sometimes even vascular growth factors [62]. By their IFN-γ production, NK cells are an early source important in T-helper 1(Th1) polarization [63] which is associated with profound vascular remodeling, unlike Th2 that leads to less profound vascular remodeling [64].

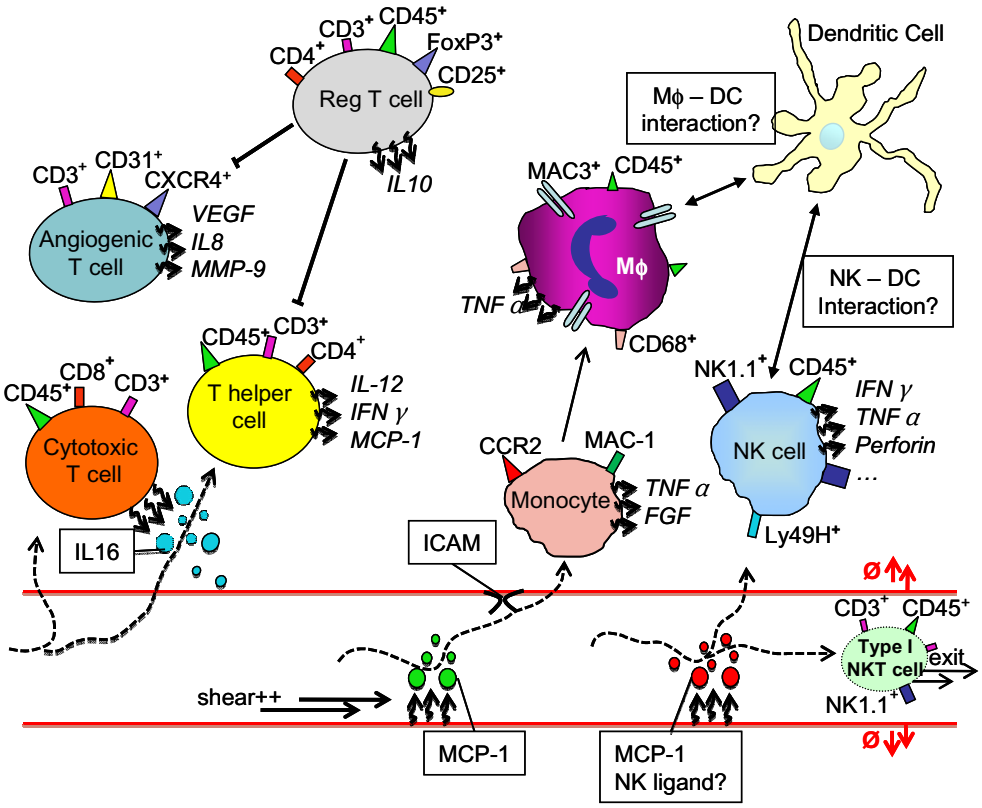

Figure 1: Involved leukocyte subsets in outward remodeling of collateral arteries. Cytotoxic CD8+ T cells invade the vessel wall early and secrete IL-16, which in turn attracts CD4+ T helper lymphocytes. These CD4+ T helper lymphocytes secrete a variety of pro-arteriogenic cytokines IL-12, IFNγ and the chemoattractant MCP-1. The latter induces of perivascular monocyte recruitment. Monocytes adhere to activated endothelium, which expresses increased levels of ICAM-1 and VCAM-1 molecules. Next, monocytes invade in the vessel wall, excrete growth factors such as Fibroblast Growth Factor (FGF) and cytokines such as TNFα. Furthermore, monocytes can differentiate towards macrophages. NK cells are also attracted by MCP-1, and probably via NK ligands that are produced upon endothelial cell stress. In the vessel wall NK cells are supposed to act as cytokine factories by secreting an array of pro-arteriogenic cytokines; IFNγ, TNFα and the cytolytic perforin. Furthermore, they might have relevant interactions with dendritic cells. Recent experiments did not demonstrate involvement of type I NK-T cells in arteriogenesis. Regulating T lymphocytes inhibit the pro-arteriogenic action of CD4+ T helper lymphocytes and of the angiogenic CD3+, CD31+, CXCR4+ T lymphocytes. The latter are attracted by hypoxia derived factors such as SDF-1 and have the ability to secrete VEGF, IL8 and MMP-9. The subtle balance of this inflammatory cascade should optimally lead to enlargement of the collateral diameter.

The Th1 response leads to rapid arteriogenesis in C57BL/6 mice, whereas BALB/c mice respond poorly due to a Th2 response. Introduction of C57BL/6 genes into BALB/c mice by the creation of a C57BL/6 x BALB/c F1 led to significant improved arteriogenesis when compared to the parent BALB/c strain [41]. This indicates that BALB/c mice are lacking a crucial factor for proper arteriogenesis.

The genetic differences between C57BL/6 and BALB/c mice also include a relevant difference in the content of the above-mentioned Natural Killer gene complex (NKC), as BALB/c mice lack a 200 kb region. This region codes for members of the Ly49 receptor family of which the C57BL/6 NKC region possesses 12 Ly49 genes, whereas the BALB/c strain only has 7 Ly49 genes. Importantly, BALB/c mice lack the NK receptor Ly49H [65] which is crucially involved in murine cytomegalovirus (mCMV) resistance, and is therefore specifically targeted against the viral mCMV glycoprotein m157 [66]. There are strong indications that the key NK cell receptor for arteriogenesis lies within this gene locus, which is normally lacking in BALB/c.

Current research now focuses on the identification of this key NK cell receptor in arteriogenesis. This will also give insight in possible therapeutic ligands to specifically stimulate NK cells for therapeutic arteriogenesis. Next, more insight in NK cell dynamics during arteriogenesis is required, including insight in local effector function. The local effector function could include perivascular cytolysis to create space for outward remodeling, but also cytokine production for modulating the local inflammatory response.

Summary

The role of leukocytes in arteriogenesis is evident, but highly complex. To design new arteriogenic treatment strategies using leukocytes, it is necessary to refine our knowledge on which subsets of leukocytes are involved in collateral formation and how. Ex-vivo activation (or inhibition) of specific inflammatory cell subpoplutions may prove beneficial for stimulation of arteriogenesis by cell therapy in the future. A major challenge for the development of these therapies remains that pro-arteriogenic leukocytes and growth factors may contribute to adverse effects such as plaque progression and neointima formation [67,68].

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Chapter 10. Local and Sustained Drug Delivery in Arteriogenesis

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Abstract: The use of targeted drug delivery is a promising approach to improve arteriogenesis using both old and novel compounds. Various vehicles to deliver and release drugs have been developed to avoid systemic side effects and to improve efficacy of drugs with a short in-vivo half life. The smallest drug delivery vehicles are nanoparticles which can either encapsulate a drug or present a drug at extending polymer tips. However, nanoparticles need a targeting modality, as they would otherwise diffuse unselectively into the tissue. Their use has therefore mainly been limited to intramuscular injections. Larger vehicles, such as microbubbles (1-8 μm) can be administered intravenously, as they are small enough to transverse through the capillary system. Subsequently, the drugs are released locally via targeted ultrasound mediated microbubble destruction, which in addition increases endothelial permeability, thereby enhancing drug uptake. Microspheres on the other hand (>10 μm) cannot traverse through the capillary system and therefore have to be injected intra-arterially after which they become lodged in the capillaries where they are degraded and subsequently release their content. All these vehicles, or combinations of, have been used in arteriogenic research and have shown promising results in both in vitro and in vivo research. Microbubbles and microspheres are currently the most advanced approach in this field. However, further research is needed in order to make targeted drug delivery in the clinical setting a reality.

Introduction

Targeted drug delivery aims to selectively transport or release drugs to the target tissue, thereby improving efficacy and reducing systemic side effects. Various strategies have been developed over the years, including the use of targeting molecules for ligands expressed on the endothelium, ultrasound to destroy drug bearing microbubbles at sites of interest and the implantation of devices which gradually release drugs over time. Despite the limited number of studies on targeted drug delivery in collateral artery growth (arteriogenesis), results so far are promising and indicate its enormous potential in the coming decades. In addition to the delivery of novel factors, targeted drug delivery might enable the use of therapeutic targets that were previously regarded obsolete because of their systemic side effects or short half life.

Examples of the latter are pro-arteriogenic chemokines and cytokines. If given systemically, effective local concentrations would inevitably be accompanied by systemic levels, possibly inducing side effects due to their manifold involvement in physiological and pathological processes. In addition, the biological effects of many cytokines are based on the creation of a chemotactic gradient, which cannot properly form when given systemically. Targeted drug delivery can be used to selectively increase local concentrations and might therefore open up new avenues of treatment. Drug delivery vehicles will possibly also allow using different forms of RNA (mRNA, siRNA, miRNA) as drugs, which are otherwise rapidly degraded when circulating freely in the blood.

In this chapter we will discuss the current stand of knowledge and recent research on targeted drug delivery to enhance arteriogenesis, particularly focusing on the use of nanoparticles, microbubbles and drug eluting stents.

Nanoparticles

Nanoparticles are particles smaller than 1 μm and can be used to achieve sustained release of factors with a short half life *in vivo* such as cytokines or chemokines. Nanoparticles have been made in various sizes and from various materials. They can be tailored to encapsulate drugs, to incorporate it into the nanoparticle structure or to 'present' the drug at the tips of extending polymer chains. Although nanoparticles themselves can be designed to meet many needs, their possibilities are limited when used for cytokine or growth factor delivery, as proteins

often cannot endure certain steps of the fabrication process involving high temperatures, extreme pH values or pressures. Nanoparticles have been used in many areas of research to deliver active factors and to prolong their presence at sites of interest [1-5] or for imaging the presence and expression of specific molecules [6-8]. Here, we will focus on nanoparticle delivery in angiogenesis and arteriogenesis.

Nanoparticles in arteriogenesis

To achieve accumulation of nanoparticles at sites of interest, the nanoparticles need to be targeted against locally expressed molecules using specific ligands, as they would otherwise remain in the circulation until they are excreted or diffused into the tissue due to their small size. Xie et al developed a ligand for the $\alpha_v\beta_3$-integrin which displayed highly specific binding properties in contrast to non-targeted control particles *in vitro* [3]. When added to cells, nanoparticles conjugated to this ligand accumulated in the cytoplasm within minutes suggesting receptor mediated endocytosis of these compounds. Moreover, these targeted nanoparticles were also tested *in vivo* by injecting them intravenously in tumor-bearing mice to determine the ability to selectively target angiogenic vessels. Lectin stainings proved incorporation of the particles into angiogenic vessels two hours after injection. However, nanoparticles also accumulated in other tissues such as liver, spleen and lung capillaries which could result in unwanted side effects, especially when the nanoparticles are loaded with drugs.

Nanoparticles can also facilitate the sustained delivery of plasmid DNA to tissue following intramuscular injection, as shown in a murine ischemic hind limb [9]. When loaded with VEGF encoding plasmids, VEGF expression after 12 days was significantly higher when compared to control or naked VEGF plasmid treatment. Biological activity was confirmed by a significant increase in BrdU positive capillaries 4 weeks after induction of ischemia. The ability of nanoparticles to locally deliver statins was assessed using nanoparticles in which either FITC or pitavastatin was entrapped in the polymer matrix. In the mouse hind limb ischemia model, FITC-labeled nanoparticles were injected intramuscularly directly after ligation. Strong fluorescent signals were localized predominantly in capillaries and arterioles with minor signal in myocytes 7 days after ligation. Fourteen days after injection, nanoparticles mainly accumulated in capillaries and arterioles and were not observed in myocytes anymore. No nanoparticles were detected at any time point in the non ischemic contra lateral hind limb or other organs such as heart, liver, spleen or kidney. Injection with pitavastatin nanoparticles resulted in increased blood flow and increased density of $CD31^+$ cells (angiogenesis) and αSMA^+ vessels (arteriogenesis) 14 days after ligation [10]. The same pitavastatin releasing nanoparticles have been further tested in a rabbit hind limb model. Preliminary results indicate that pitavastatin bearing nanoparticles increase angiographically visible collaterals and prevent exercise induced ischemia [11].

Despite these encouraging results, nanoparticles have certain important disadvantages. Their loading capacity is smaller than that of microbubbles or microparticles. Hence, the nanoparticle doses need to be increased accordingly to ensure efficient drug delivery. Most importantly, recent studies have raised safety concerns regarding nanoparticle use as they may impair cellular function [12], increase production of reactive oxygen species [13] and potentially have carcinogenic effects [14-16].

Microbubbles

Microbubbles are fast and reliable carriers for targeted drug delivery. Their properties can be easily adapted to accommodate specific needs regarding release or targeting.

Microbubble construction and types of microbubbles

Microbubbles are small (1-8 μm) gas filled vesicles which can be made from proteins, lipids or biocompatible polymers (biopolymers). The latter two have mainly been used for drug delivery, as their properties can be easily modified to adjust e.g. degradation kinetics, microbubble size, or to incorporate targeting modalities on the outside of the microbubble.

Lipid and biopolymer microbubbles behave differently when ultrasound is applied (Fig. **1**). Lipid based microbubbles oscillate during application of ultrasound with a very low to low acoustic power, resulting in ultrasound scattering ideal for imaging. When ultrasound with a higher acoustic power is applied, the microbubble undergoes forced expansion and compression leading to microbubble destruction and release of the gas core. Biopolymer based microbubbles are stiffer and are therefore hardly deformed when subjected to ultrasound. When the acoustic power exceeds a certain threshold, cracks appear through which the incorporated gas can escape. A further increase in the applied acoustic power leads to complete microbubble disintegration. The choice whether to use lipid or biopolymer based microbubbles therefore highly depends on the actual requirements and needs. As lipid based microbubbles are able to oscillate at low intensity, they are ideal for imaging and their lipid bi-layer enables them to fuse with target cells to release their content intracellularly. Biopolymer based particles are more robust and, contrary to lipid based microbubbles, their content is often released immediately when subjected to high acoustic powers. This decreases the risk of undesired drug release before high intensity ultrasound is applied to release all the encapsulated drugs in one single burst [17].

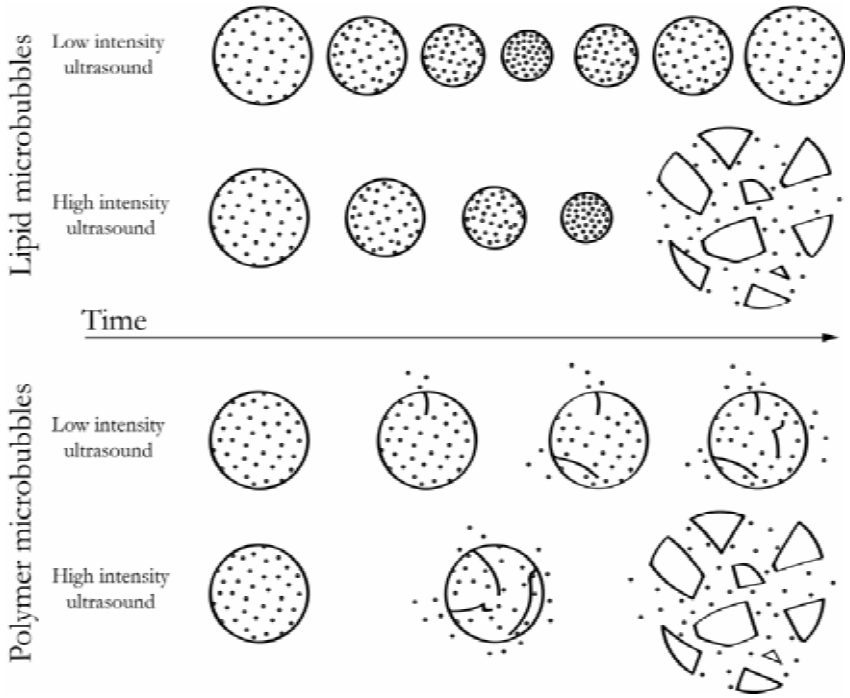

Figure 1: Schematic representation of ultrasound mediated microbubble destruction. Lipid microbubbles only oscillate when low intensity ultrasound is applied and implode when higher intensity ultrasound is applied. Polymer based microbubbles do not oscillate under low intensity ultrasound but cracks do appear in the microbubble shell through which the content can escape. High intensity ultrasound leads to complete microbubble destruction when a certain threshold is reached. Adapted from Hernot and Klibanov, Advanced Drug Delivery Reviews, 2008 [17].

One of the biggest advantages of microbubbles is their ability to traverse through the capillary system of the lungs and to reach the whole body when injected intravenously. Using low intensity ultrasound the microbubbles can be followed before destruction and content release. During destruction of the microbubbles the endothelium is often perforated by the small 'jets' which are produced during microbubble destruction. In combination with the increased

endothelial leakage due to the ultrasound application, local drug uptake might be further enhanced [18,19].

Microbubble treatment in combination with ultrasound

The use of ultrasound and 'non loaded' microbubbles by itself might induce an arteriogenic response as this might lead to increased activation of the endothelium and subsequent leukocyte extravasation resulting in increased arteriogenesis. As previously shown, ultrasound treatment of rat skeletal muscle after injection of microbubbles results in an increase in the number of arterioles and an increase in blood flow when compared to sham treated muscle [20]. After arterial occlusion, treatment with ultrasound and microbubbles increased blood flow significantly at various time points and resulted in a restoration of blood flow during reactive hyperemia to normal levels within 7 days. Furthermore, the number of arterioles as assessed by smooth muscle actin staining per muscle fiber was significantly upregulated 14 and 28 days after intervention [21]. This effect might be due to the enhanced extravasation of VEGF producing inflammatory cells. Immunohistochemical analysis of muscles treated with ultrasound and microbubbles after arterial occlusion revealed a greater leukocyte ($CD45^+$) cell count 3 and 7 days after intervention. These cells included $F4/80^+$ (macrophages) and $CD3^+$ (T-lymphocytes) cells which were both positive for VEGF staining [22].

Microbubble treatment in combination with ultra sound and systemic administration of arteriogenic factors

As described above, the use of microbubbles results in an increased permeability of the endothelium and vascular leakage which might be used to enhance the diffusion and/or uptake of arteriogenic factors by the tissue. Mukherjee et al. showed that this was feasible in a rat model where VEGF was infused intravenously and ultrasound was subsequently applied to the chest [23]. The result was an increased uptake of recombinant VEGF by the endothelium (8-fold), which was further enhanced to a 13-fold increase when combined with a contrast agent. In addition, microbubbles in combination with ultrasound can also be used to enhance the local uptake of plasmids encoding arteriogenic factors [24]. The expression of transgenes was highly increased for four days after treatment before declining rapidly. Repetition of the procedure resulted in a second peak which displayed similar decay effects, thereby providing a reliable method to regulate transgene expression. Microbubbles in combination with ultrasound can also be used to improve cytokine and growth factor delivery [25]. G-CSF injection at six days after ligation of the femoral artery resulted in enhanced vascularization 28 days after ligation, while combined treatment with microbubbles and ultrasound further improved vascularization. However, as enhanced uptake of G-CSF by the ultrasound treated tissue was not shown, it remains to be elucidated whether the observed increase is due to two different processes or a synergy of G-CSF administration and ultrasound treatment.

Microbubble induced endothelial perforation can also be used to enhance the extravasation of nanoparticles. In an *ex vivo* study the effect of different injection sites and ultrasound variables on microbubble mediated extravasation of nanoparticles to muscle was assessed [26]. The extravasation points created by the microbubbles and the subsequent extravasation of nanoparticles through these openings were imaged. A significant difference in extravasation was not observed when different injection sites (venous vs. arterial) were used, however shortening the injection time from 40 s to 10 s did increase the amount of delivered nanoparticles significantly. Furthermore, it was observed that an ultrasound pulse interval of 5-10 s used for microbubble destruction yielded the highest overall extravasation of nanoparticles. This strategy was also tested in a hind limb ischemia model where a combination of FGF2-bearing nanoparticles, microbubbles and ultrasound resulted in an increase in both the number and overall luminal expansion of arterioles [27]. The delivery of nanoparticles to the heart was assessed after intravenous administration of nanoparticles [28]. Microbubbles in combination with ultrasound treatment highly enhanced nanoparticle delivery to the heart, which increased with rising ultrasound peak pressures. However, higher peak pressures also increase the risk and amount of epicardial hemorrhage. It remains therefore imperative to continuously balance the benefits of nanoparticle delivery and the detrimental effects of ultrasound treatment.

Arteriogenic factors can also be incorporated in the microbubble itself and subsequently released when the microbubble is destroyed using ultrasound. Treatment of severe chronic hind limb ischemia in rats with microbubbles bearing VEGF resulted in a significant increase in vessel density and tissue perfusion 14 days after delivery of microbubbles when compared to non-treated rats [29]. Microscopic analyses after injection of GFP encoding plasmid bearing microbubbles showed efficient transfection of arterioles, capillaries and muscle fibers adjacent to capillaries. After 14 days, the GFP signal was mainly located in the endothelium of small to medium sized arterioles. In contrast to the aforementioned studies, the authors did not find a beneficial effect of treatment with ultrasound in combination with microbubbles bearing GFP plasmid only. This might be due to differences in the acoustic power applied, dosage/composition of microbubbles or conditions under which perfusion restoration was assessed (resting conditions vs. vasodilatation).

Recently, Chappell and colleagues aimed to elucidate the mechanism underlying the pro-arteriogenic effect of microbubble and ultrasound treatment alone [30]. Rats and mice were subjected to bilateral ligation of the femoral artery. Subsequently, microbubbles were injected intravenously and one of the hind limbs was treated with ultrasound. Both, rat and wild-type mice hind limbs displayed enhanced perfusion restoration compared to contralateral controls up to 28 days after surgery. Ultrasound application furthermore led to increased CD18 mediated recruitment of $CD11b^{+}$ mononuclear cells as no effects of ultrasound were observed in chimeric mice carrying CD18 -/- bone marrow. These findings are consistent with earlier experiments in ICAM-1 -/- and Mac-1 -/- mice [31] and suggest an enhancement of leukocyte adhesion and extravasation as the primary mechanism of microbubble-induced stimulation of arteriogenesis.

Microbubble treatment in combination with ultra sound and systemic administration of cells

In light of the results mentioned above, ultrasound in combination with microbubbles could also be used to enhance the efficacy of cell transplantation. Usually, intramuscular injection is superior to intravenous delivery with regards to cell number reaching and remaining at the site of interest. However, combination of intravenous cell injections with microbubbles and ultrasound can result in arteriogenic effects equal to intra muscular injection of cells [32]. *In vitro* experiments indicate that this enhanced cell homing might be due to increased activation of the endothelium as a result of treatment with ultrasound and microbubbles. This effect seems to be mediated by platelets, as their depletion from the culture medium completely abolished endothelial activation after ultrasound stimulation as measured by P-selectin and ICAM-1 expression. Treatment with ultrasound and microbubbles might also exert additive beneficial effects on intramuscular injection of cells, as this resulted in an increase in blood flow and capillary density when compared to intramuscular injection of cells alone [33]. The use of ultrasound in combination with microbubbles to augment intravenous cell delivery was also tested in a hamster cardiomyopathy model. Either treatment alone did not significantly improve outcome. However, combination of both treatments resulted in a significant improvement in tissue perfusion and cardiac function [34].

Instead of administrating purified cell fractions, another option could be the injection of “cell beads” consisting of a tissue or cellular core encapsulated by a porous membrane, which allows the diffusion of soluble factors but prevents immune reactions leading to the rejection of the transplanted tissue by the host. First studies using such an approach have shown promising results both *in vitro* and *in vivo* indicating that this ‘of the shelf’ product can be used to achieve sustained long term delivery of therapeutic factors without the risk of an immune response [35].

Imaging using microbubbles in combination with ultrasound

Recent advances in targeted imaging may offer new ways to improve the efficacy of targeted delivery. *In vitro* adhesion of microbubbles targeted to ICAM-1 on the endothelium increases with increasing levels of inflammation indicating the possibility of targeting endothelial inflammation using targeted microbubbles [36]. *In vivo,* α-integrin targeted and contrast filled

microbubbles have been used to image endothelial integrin expression in rat hind limbs after femoral artery ligation. Adductor muscle integrin signal peaked after 4 days and signal intensity was higher in FGF2 treated rats which also showed increased perfusion recovery [37]. Activated neutrophils and VCAM-1 and α-integrin expression after femoral artery ligation have been investigated using targeted microbubbles in mice. An increase in signal intensity was observed which corresponded to the increase observed using immunohistochemical staining for these markers [38].

These examples of targeted microbubbles also create new possibilities for targeted drug delivery in general, as drugs can not only be directed to specific areas, but even to certain cell types expressing the respective target molecule. The expression of integrins on the activated endothelium during angiogenesis has been imaged non-invasively for example in both myocardial infarction and hind limb ischemia models [39-41]. Integrins and other molecules expressed on the endothelium of the developing collateral, such as ICAM-1, P-selectin and other activation markers, are therefore likely to form suitable and promising targets for targeted drug delivery in arteriogenesis [42].

Microspheres (diameter > 10 μm)

In addition to incorporating targeting molecules or the application of ultrasound to locally release arteriogenic factors using microbubbles, microspheres can be used to deliver arteriogenic compounds. Due to their larger diameter (> 10 μm) microspheres are "lodged" in the microcirculation where they are degraded and subsequently release their content. As the capillaries are blocked by the microspheres, their dosage is an important issue to be taken into account. While lower numbers do not lead to significant changes in oxygen supply, high microsphere numbers can lead to embolism and tissue necrosis, therapeutically used in oncological studies [43].

In an ischemic hind limb model in rabbits, gelatin hydrogel microspheres were injected into the internal iliac artery bearing bFGF 21 days after ligation [44]. Detection of the radioactively labeled microspheres revealed that microspheres <10 μm were distributed throughout the body, while microspheres > 29 μm were mainly detected in the ischemic hind limb (~77%). Collateral flow, angiographic score and $\alpha\text{-SMA}^+$ vessel density were significantly improved 28 days after injection when compared to PBS injection or intramuscular injection of bFGF bearing microspheres. Larger (59 μm) bFGF bearing hydrogel microspheres were also used to evaluate their efficacy in treating ischemia in ischemic skin flaps. Delivery of bFGF using microspheres improved neovascularization and reduced necrosis when compared to bolus injection of bFGF, indicating that sustained delivery using microspheres could enhance viability of the skin flap [45]. In a recent study it was shown that the sustained release of erythropoietin using an intramuscular injection of erythropoietin loaded microspheres improves blood flow eight weeks after surgery. Remarkably, the treated animals did not have enhanced blood flow until four weeks after treatment when compared to PBS treated animals [46].

Recently, G-CSF has been shown to stimulate arteriogenesis when incorporated into gelatin hydrogel microspheres, resulting in a higher perfusion recovery and an increased number of arterioles when compared to controls [47].

Drug eluting stents

Since the development of intracoronary stents, it has been hypothesized that they could also be used as drug delivery platforms [48]. Research exploring the possibility to do so has mainly focused on drug eluting stents which could decrease or even prevent restenosis and the resulting pathology and morbidity amongst cardiovascular patients. In general, drug eluting stents are composed of a metal stent coated with biocompatible polymers which are degraded over time, thereby releasing the drug which is incorporated inside the polymer. More recently, whole stents have been made of degradable polymers leading to complete disintegration of the stents within a few months. Several studies have shown the decrease in restenosis rate after implantation of drug eluting stents [49-51], however the safety and overall benefit of drug eluting stents remain a point of discussion [52,53].

Stents are usually not regarded as a delivery platform for the improvement of collateral vessel growth. In addition, several studies have shown that the collateral circulation might even be at risk after coronary stenting, as collateral vessel development is dependent on the altered blood flow due to a stenosis. One of the first studies to describe such a detrimental effect reported a more then 50% decrease in collateral flow index directly after stent placement [54]. Placement of drug eluting stents in patients with chronic total occlusion results in improvement of stenosis severity but also induces a rapid reduction of collateral flow putting the patients at higher risk for future ischemic events [55]. While collateral flow indices were diminished in >90% of patients receiving a drug eluting stent, only 55% of patients receiving a bare metal stent displayed insufficient collateral development [56]. Finally, drug eluting stents have also been used to deliver pro-arteriogenic compounds to the developing collateral circulation. After femoral artery ligation in rabbits, TGF-β1 eluting, bare metal or polymer coated stents were deployed in the external iliac artery. TGF-β1 release from the stents led to an increased collateral conductance when compared to rabbits treated with a control stent or a single TGF-β1 bolus. This was supported by higher vascular proliferation rates. This study indicates that stents might serve as intra-arterial delivery platforms for cytokines [57].

Loading capacity and the targeted delivery of drugs directly to the intima might be enhanced using a novel stent design, which consists of honeycombed strut elements filled with stacked layers of drugs and polymers (Fig. **2A**). This unique construction enables controlled and sustained release of drugs in various situations which require different pharmacokinetics and enable the targeted delivery of both vessel wall and vessel lumen with different drugs (Fig. **2B**) [58]. When tested in clinical trials, it was found that such a device was not only safe but that its prolonged release kinetics (30 days) resulted in a lower in-stent late loss, less volume obstruction and a reduced number of major adverse cardiac events when compared to a shorter release time span (10 days) or bare metal stents [59].

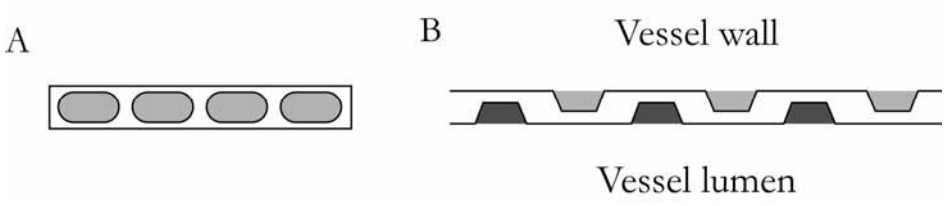

Figure 2: A stent with honeycombed strut elements has the same overall design as a normal stent. However, the surface of the stent has small indentations which are filled with drugs which are released over time (A). As the small indentations are placed on both sides of the stent, the vessel wall and vessel lumen can be targeted with different drugs (B).

Conclusion

The use of targeted drug delivery in arteriogenesis is a relatively new but fast developing field of research. The research described in this chapter shows that targeted drug delivery is a promising tool to circumvent many of the problems often related with the therapeutic application of arteriogenic factors, such as a short half life of the drugs or severe systemic side effects when used in high concentrations.

Two avenues of targeted drug delivery seem most promising: the use of microbubbles in combination with ultrasound and the lodging of larger sized microspheres in the microvasculature. The former has been the focus of many studies and results indicate high efficacy with almost no side effects, although concerns remain about the extensive use of ultrasound needed to destroy the microbubbles. However, a big advantage of microbubbles is that they can be administered intravenously as they are able to traverse the lungs because of their small size.

The use of microspheres does not involve a secondary technique such as ultrasound to release the encapsulated drugs and therefore does not have the risk of damaging tissue. However, microspheres have to be administered intra-arterially and locally as they are too large to travel through the pulmonary microcirculation and their efficacy is based on local lodging in the capillaries.

Although many important issues yet need to be solved, the results obtained so far clearly illustrate the high potential of targeted drug delivery for therapeutic stimulation of arteriogenesis.

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Chapter 11. Collateral Artery Growth in Man, from Assessment to Stimulation

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Abstract: In patients with coronary artery disease, collateral arteries reroute the blood flow around coronary stenoses to the myocardial area at risk and preserve myocardial tissue perfusion. Several diagnostic modalities have been introduced over the years to quantify the capacity of the coronary collateral circulation. Although there is some controversy, many studies have demonstrated the association between sufficient collateral arteries and improved clinical outcome. Therefore, the therapeutic stimulation of collateral artery growth seems an appealing concept to improve clinical outcome of patients with coronary artery disease. This chapter discusses various modalities to quantify the capacity of the coronary collateral circulation. The relevance of collateral arteries in patient with coronary artery disease is discussed in more detail, and therapeutic options for the stimulation of collateral artery growth are reviewed. Finally, future perspectives are outlined.

Introduction

In the 1950s, Fulton demonstrated unequivocally the presence of small arteriolar vessels connecting different vascular territories of the normal human heart, using high resolution post-mortem angiography [1,2]. His findings definitively ended the long lasting dispute over the existence of arteriolar connections in the normal human heart. Fulton's study was an extrapolation of previous studies by Baroldi *et al*., demonstrating expanding interconnections between coronary arteriolar networks upon coronary occlusions, using cast-corrosion models [3]. These observations formed the basis for the concept of arteriogenesis, the remodeling of pre-existing arteriolar connections into large collateral arteries upon arterial stenosis. Collateral arteries divert blood flow around the arterial stenosis, thereby partially or completely preventing ischemic damage. Depending on the location of the stenosis, collateral arteries may alleviate symptoms e.g. angina pectoris in coronary artery disease (CAD) and ischemic leg pain in peripheral artery disease (PAD). Although the clinical relevance of the human coronary collateral circulation in patients with CAD has been well recognized, it has also been a matter of debate. Whether the coronary collateral circulation is associated with improved outcome in patients with CAD is substantially influenced by the patient population, the modality to measure the capacity of the collateral circulation and the definition of endpoints. The focus of this chapter is to discuss diagnostic modalities to quantify the capacity of the coronary collateral circulation, the clinical relevance of arteriogenesis in patients with CAD, and to summarize current knowledge on the therapeutic modification of collateral artery growth.

Assessment of the coronary collateral circulation

When one of the coronary arteries becomes gradually obstructed, arteriolar connections between the donor artery (also called, the contralateral vessel) and the recipient artery (also called the ipsilateral vessel) expand and are transformed into large collateral vessels that divert the blood flow to the jeopardized vascular territory. Several methods have been developed to quantify collateral flow (Table 1), which all are based on the two assumptions that (1) blood flow distal to an arterial stenosis is the sum of the blood flow derived from the recipient coronary artery and collateral flow, and (2) that the blood flow distal to a total occluded coronary artery is completely dependent on collateral flow.

Angiography

In the cardiac catheterization laboratory, angiography is the most widely used technique to assess the capacity of the collateral circulation in patients. In a landmark study, Rentrop *et al*.

showed that visualization of the collateral vessels depends on the existence of a pressure gradient between the different vascular territories [4]. In some of the CAD patients, collateral arteries are spontaneously visible on angiography. However, these so called "spontaneously visible" collateral arteries represent only a small fraction of the total collateral circulation. After creating a maximal pressure gradient by balloon coronary occlusion of the recipient artery, the so called "recruitable" collateral arteries become apparent by simultaneously injection of contrast in the donor artery, using a second arterial catheter. Using this approach, collateral flow can be graded according to the Rentrop classification: 0 = no filling; 1 = small side branches filled by collateral vessels; 2 = partial epicardial filling of the recipient artery by collateral vessels; 3 = complete epicardial filling of the recipient artery by collateral vessels [4]. In the absence of a total coronary occlusion, accurate grading of collateral flow thus requires a second arterial catheter for contrast injection into the donor artery during balloon coronary occlusion of the recipient artery. In clinical practice, a great advantage of the Rentrop classification is its wide availability. However, its accuracy is limited to documentation of spontaneous visible collateral vessels with a diameter >100μm [5,6]. Therefore, the Rentrop classification is a rather crude method to assess the total capacity of the collateral circulating. Another angiographic method to quantify the collateral flow is contrast washout collaterometry. Contrast washout collaterometry is based on the inverse relation between collateral flow and the time to contrast clearance injected distally into the balloon occluded recipient artery. Whereas an angiographic Rentrop score of 2 obtained during balloon coronary occlusion detects sufficient collaterals with 71% sensitivity and 68% specificity, a washout time of ≤11 heart beats accurately determines sufficient and insufficient collaterals with 88% sensitivity and 81% specificity [7].

Intracoronary hemodynamics

As compared to angiography, intracoronary blood flow and pressure measurements using guidewires equipped with Doppler crystal and/or pressure sensors enable more accurate quantification of the collateral flow. These wires facilitate assessment of collateral hemodynamics directly in the epicardial segments of the coronary arteries. The collateral flow can be qualitatively assessed with a Doppler-wire as an antegrade, retrograde or bidirectional flow velocity signal, depending upon the position of the tip of the guidewire in relation to the collateral flow direction [8-10]. In general, two methods are used to analyze the hemodynamic data obtained with these wires. The flow velocity-derived collateral flow index (CFI_v) is the ratio between the coronary blood flow velocity (CFV) during intracoronary balloon occlusion of the recipient artery, and the CFV at the same location during vessel patency, following successful percutaneous coronary intervention (PCI) ($CFI_v = CFV_{occ} / CFV_{pat}$) [10]. In a similar way, the pressure-derived collateral flow index (CFI_p) is determined by simultaneous measurement of the mean aortic pressure (P_{ao}), the mean central venous pressure (P_v) and the mean distal coronary pressure during balloon occlusion of the recipient artery (P_{occ}), ($CFI_p = (P_{occ} - P_v)/(P_{ao} - P_v)$). Compared to angiographic grading, the intracoronary hemodynamic measurements have been demonstrated to be a much more reliable method for functional quantification of the coronary collateral flow [10]. CFI_p and CFI_v correlate well with each other (CFI_v vs. CFI_p: $r = 0.8$), and both methods are the reference methods for the assessment of the coronary collateral flow in clinical practice [11]. Importantly, each method is dependent on different physical laws. While the CFI_v is affected by vessel diameter, tortuosity, branching and position of the tip of the guidewire, the CFI_p is more influenced by the microvascular function [13] and conditions of elevated transmural stress, such as increased left ventricular end-diastolic pressure [14,15]. In the clinical setting, CFI_p is easier to assess and has a better reproducibility, as the signal is not critically dependent upon the position of the tip of the wire in the epicardial coronary segment. Recently, direct volumetric measurements of coronary flow using thermo-dilution have emerged as another invasive method that quantifies the collateral flow. During continuous low-rate infusion of saline at room temperature, assessment of the infusion rate of saline, the temperature of the saline at the tip of the infusion catheter, and the distal blood temperature allow the quantification of coronary flow and resistance, rather than flow velocity [16]. Future studies are warranted to determine the feasibility and accuracy of this approach.

Table 1: Methods for Assessment of Collateral Flow (adapted and modified from Berry *et al.*[12])

Modality	Quantification	Strengths	Weaknesses
Angiographic			
Angiographic collateral Degree	Visual	Easy	Limited resolution
Angiography – computer assisted	Visual – computer assisted	Easy; time consuming	Not routinely performed
Angiographic collateral degree	Rentrop's grade	Easy; standard method	Angiographic assessment for recruitable collaterals requires a double injection technique; a second arterial puncture is required for injection into the donor artery prior to, and during balloon inflation in the recipient artery (culprit) artery
Angiographic collateral Degree	Collateral connections Score	Easy	Optimal angiographic views required to avoid foreshortening
Angiographic coronary Flow	TIMI frame count Fourier spectral analysis Cineangiographic modelling Washout collaterometry	Washout collaterometry does not require a second arterial puncture, is easy to do	Dedicated software required for Fourier spectral analysis; subject to the effects of variation in contrast injection and alterations in distal microvascular resistance
Physiological			
Intracoronary hemodynamics	Pressure-derived collateral flow index	Validated	Expensive; relevant technology required as well as appropriate training
Intracoronary hemodynamics	Flow velocity-derived collateral flow index	Validated	Expensive; relevant technology required as well as appropriate training
Perfusion imaging			
Contrast echocardiography	Myocardial perfusion	Easy; contrast may be administered by intra-coronary or intravenous injection	Invasive access still required to demonstrate a chronic total occlusion, or if antegrade flow is present, to obstruct flow by transient balloon occlusion
MRI	Coronary blood flow and myocardial perfusion	Accurate assessment of myocardial perfusion; may also provide information on myocardial viability	Limited resolution
PET	Myocardial perfusion	Information on perfusion and metabolic function	Expensive; off-line analysis
Myocardial scintigraphy	Myocardial perfusion	Assessment of myocardial perfusion	No direct assessment of collateral blood flow

MRI, Magnetic resonance imaging; PET, positron emission tomography; TIMI, thrombolysis in myocardial infarction.

Non-invasive assessment of the collateral circulation

Several non-invasive strategies have been introduced over the years to assess the capacity of the coronary circulation i.e. myocardial contrast echocardiography (MCE), positron emission tomography (PET) and myocardial scintigraphy. The major disadvantage of these methods is that they require coronary angiography to either detect a complete occluded coronary artery, or to perform intracoronary balloon occlusion of the patent culprit coronary artery for accurately measuring collateral function (mL/min/g of myocardial tissue). Vogel *et al.* investigated the feasibility of MCE with systemic administration of contrast, to assess the coronary collateral flow during intracoronary balloon occlusion. A close relationship was found between the absolute collateral-derived myocardial blood flow, as measured by MCE, and CFI_p [15]. PET is an interesting research tool for quantification of the collateral flow; however, the technique is not very practical in a clinical setting [17]. Myocardial perfusion imaging after ^{99m}Tc-Sestamibi radionuclide injection has also been used to quantify collateral flow by evaluation of the extent of the perfusion defect during intracoronary balloon occlusion of the recipient artery [11]. Both magnetic resonance imaging (MRI) and multi-slice computed tomography (CT) potentially can be used for assessment of the collateral circulation; however, the question remains whether small collateral connections can be delineated by these techniques in all myocardial segments.

The clinical relevance of the coronary collateral circulation

When a critical stenosis is present in one of the main coronary arteries, the perfusion of the area behind the stenosis is largely dependent on collateral flow. In case of insufficient collaterals, this myocardial area may be at risk for ischemia or infarction. Although many studies have shown that sufficient collateral arteries are associated with improved clinical outcome in patients with CAD, other studies have shown contradictory results (reviewed by Berry *et al*. [12]). The reason for these conflicting results is probably related to differences in methodology, such as patient population, the modality to measure the capacity of the collateral circulation, and the definition of endpoints.

Collateral vessels in healthy individuals

It has long been assumed that coronary arteries are end-arteries in the absence of stenosis; however, even in entirely normal post-mortem human hearts, the presence of arteriolar connections between different vascular territories have been demonstrated [3]. At the cardiac catheterization laboratory, collateral flow has been detected during brief balloon coronary occlusion in patients with angiographically normal coronary arteries. In 20-25% of these patients, the collateral flow was sufficient to prevent electrocardiographic signs of myocardial ischemia [18]. Thus even in the absence of CAD, the collateral circulation is capable to protect against myocardial ischemia during brief coronary occlusion in some patients.

Collateral vessels in stable CAD patients

In patients with stable CAD, many studies have shown an association between the collateral flow and improved clinical outcome. Sufficiently developed collateral arteries that prevent signs of myocardial ischemia (angina pectoris and electrocardiographic changes) during brief coronary occlusion are present in about one-third of patients with CAD [19]. In a patient study encompassing 100 patients with a total coronary occlusion, Werner *et al*. found an association between the collateral flow and the preservation of the regional left ventricular myocardial function [13]. Furthermore, it has been shown in CAD patients, that patients with a low CFI_p (<0.23) have more ischemic events during one-year of follow up, as compared to patients with a high CFI_p [20]. Moreover, in another large cohort of CAD patients, a clear reduction was observed in long-term cardiac mortality in CAD patients with a higher CFI_p (>0.25) [19]. Importantly, collateral arteries are an integrated part of the coronary circulation, and therefore, may exert different effects depending on their functionality and on the context. Well developed collaterals respond to vasodilators, such as nitroglycerin and adenosine, whereas the immature collaterals are less responsive [21]. Seiler *et al*. evaluated the incidence

of "coronary steal", i.e. the redistribution of blood flow during vasodilation away from the area in need. Microvascular vasodilation, e.g. during exercise or pharmacological stimulation, may lower the pressure in the donor artery, resulting in a decreased blood flow via the collateral arteries to the collateral-dependent recipient artery. Coronary steal occurs in 10 percent of the patients with a partial coronary stenosis and in one third of the patients with a chronic total coronary occlusion [13,22]. In the patients with a partial coronary stenosis, Seiler *et al*. further showed a positive association between the capacity of the collateral flow and the occurrence of steal from it. Patients with increased coronary steal during hyperemia showed less often signs of ischemia (i.e. angina and electrocardiographic changes) during intracoronary balloon occlusion, as compared to patients without coronary steal. The CAD patients with a low collateral flow and without coronary steal during hyperemia, seem to be more at risk for development of ischemia during hyperemia, as the collateral flow may be just capable of preventing ischemia at rest [22]. Taken together, collateral artery growth is obviously a protective mechanism against progressive atherosclerotic disease. However, it has to be acknowledged that the presence of collateral arteries is not always beneficial. For instance, in patients with a well-developed collateral circulation, collateral vessels may divert blood flow away from an ischemic area (collateral steal), and therefore, provoke ischemia [23]. This may explain the potential pro-ischemic effects of nifedipine in angina pectoris [24]. Furthermore, a well developed collateral circulation may adversely affect outcome in the setting of PCI, in view with its association with an increased risk for restenosis [25].

Collateral vessels in patients acute with myocardial infarction

In patients with an acute myocardial infarction, the vascular lesion progresses too fast or the collateral circulation develops too slowly to compensate for the abrupt obstructed antegrade coronary flow. Nevertheless, several studies have demonstrated a beneficial role of collateral arteries, which are present in approximately 10-35 percent of the patients presenting with acute myocardial infarction [26-28]. Patients with a higher Rentrop's grade (2-3) have lower incidence of post-infarct heart failure, less occurrence of hemodynamic instability, improved myocardial tissue perfusion and smaller infarct size, as compared to the patients with a lower Rentrop's grade (1-2). Moreover, mortality is reduced in patients with acute myocardial infarction that have well developed collateral arteries (4% vs. 9% at 6 months; $P = 0.01$). Although the presence of collateral arteries at the time of primary PCI is related to better outcome, it is important to note that patients with acute myocardial infarction display normal patency of epicardial arteries after successful primary PCI. Therefore, after primary PCI the growth of collateral arteries does not occur because the trigger for arteriogenesis, a pressure deficit between a donor artery and the recipient artery, is not present. In case of failure of reperfusion therapy, angiographic studies have shown that collateral vessels that are initially absent can become apparent within 10-14 days following sustained coronary occlusion after acute myocardial infarction [29,30]. Survival after acute myocardial infarction without successful restoration of the epicardial blood flow is related to recruitability of the collateral arteries [31].

Predictors of collateral artery growth

Most studies, investigating factors important for arteriogenesis, report the severity of stenosis as the most important predictor of collateral flow [32,33]. Piek *et al*. further investigated the predictive value of several clinical and angiographic variables for collateral flow in 105 patients with single vessel CAD. Duration of angina, the severity of stenosis, and proximal lesion location were found to be independent factors that were positively associated with the collateral flow [32]. With regard to risk factors associated with CAD, it has been suggested that hypertension may display positive effects on collateral artery growth [7], while metabolic disorders may inhibit this process; however, evidence for this is scarce. In a large patient study, no difference was found in CFI_p between the non-diabetic patients ($n = 437$) and the diabetic patients ($n = 89$). On the other hand, total serum cholesterol showed a trend toward higher values in the group with a low CFI_p ($n = 307$) as compared to the group with a high CFI_p ($n = 143$). However this trend did not reach statistical significance ($P = 0.09$) [33,34]. Remarkably, a large heterogeneity exists in the extent of collateral flow, even amongst

patients without CAD, or, with similar severity of CAD [18,32]. The reason for this is unclear and is probably related to a certain genetic background or environmental factors.

Therapeutic augmentation of collateral artery growth

The beneficial role of collateral vessels in patients with CAD justifies the search for therapeutic strategies aimed at augmentation of collateral artery growth. Since collateral artery growth is induced by an increase in shear stress, physical exercise would theoretically be a stimulator of collateralization. Furthermore, several growth factors as well as other cytokines and proteins that influence collateral artery growth have been shown to augment collateralization in experimental models (Table 2) [35]. Therefore, most of the factors have been tested in clinical trials.

Exercise

There is emerging evidence that physical exercise augments the collateral flow in stable CAD patients [36]. Zbinden *et al*. assessed the collateral flow in the culprit coronary artery and in a normal coronary artery in 40 CAD patients undergoing elective PCI. The patients were divided in an exercise training group and a sedentary group. After 3 months, a dose-response relation was found between the collateral flow augmentation and the exercise capacity gained [37]. Recently, external counterpulsation has been investigated in patients with CAD, as a non-invasive alternative approach to active physical exercise. External counterpulsation is proposed to stimulate collateral artery growth via an increase in shear stress. Interestingly, the CFI_p significantly increased in the CAD patients treated by external counterpulsation, whereas no change was observed in the control group [38].

Colony-stimulating factors

After granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to increase the lifespan of monocytes/macrophages at sites of developing collateral arteries in an experimental setting, GM-CSF evolved as a potential stimulator of collateral artery growth [39]. In 2001, Seiler *et al*. randomized 21 patients with extensive CAD to treatment with GM-CSF or placebo. The authors showed an increase in collateral flow in the GM-CSF group as compared to the placebo-group [40]. In their most recent study, two patients of the treatment group developed an acute coronary syndrome, and the study was prematurely stopped [41]. Although GM-CSF treatment may lead to enhanced collateral flow in CAD patients, safety issues remained a concern. The effect of GM-CSF administration was also assessed in PAD patients; however, no effect of GM-CSF treatment was found on the walking distance or exercise capacity [42,43]. Aside from GM-CSF, the role of granulocyte colony-stimulating factor (G-CSF), as a stimulator of collateral artery growth has also been investigated. It has long been assumed that G-CSF may potentiate collateral artery growth by increasing stem cell mobilization from the bone marrow into the peripheral blood. These cells were thought to stimulate collateralization by their paracrine effects. However, it has been shown that the stimulating effects of G-CSF are probably in part also mediated by the stimulation of leukocyte adhesion [52]. Very recently, Meier *et al.* showed in a randomized patient study that a 2-week period with subcutaneous G-CSF treatment increases the CFI in patients with chronic stable CAD, as compared to placebo treatment ($P < 0.0001$), suggesting that G-CSF treatment may enhance arteriogenesis in CAD patients [44].

Fibroblast growth factors (FGF)

The effect of several fibroblast growth factors on collateral artery growth have been tested in clinical trials, as this factor not only induces endothelial cell proliferation but also smooth muscle cell proliferation and hence, potentially stimulates collateralization. Interestingly, Laham *et al*. implanted FGF-2 loaded pellets in territories that could not be revascularized in patients undergoing coronary artery bypass grafting (CABG). After 3 months, the perfusion defect decreased in the group receiving the highest dose of FGF-2 [50]. In contrast, the largest

controlled clinical trial using FGF-2 in 337 patients with CAD did not show an effect on exercise tolerance and myocardial perfusion imaging [49]. In PAD patients, the results of the TRAFFIC study showed an increased walking time at 90 days after FGF-2 treatment, suggesting a pro-arteriogenic effect [47]. The pro-arteriogenic effects of FGF-4 are less well investigated. In patients with stable CAD, a serotype 5 adenovirus encoding for the FGF-4 gene was administered intra-coronary, to achieve prolonged FGF-4 release into the coronary circulation. Initial results showed a promising trend towards an improved myocardial perfusion after 8 weeks of follow-up, as measured with single-photon emission computed tomography (SPECT) [48]. However, a larger follow-up study was prematurely abrogated, as the change in the primary endpoint at interim analysis at 12 weeks would not reach significance [53].

Table 2: Clinical Trials for the Stimulation of Collateral Artery Growth using Growth Factors and Other Proteins (adapted and modified from Schirmer *et al*.[35])

Study	Disease	Substance	Route of administration	Effect of treatment versus control
Seiler *et al.* [40]	CAD	GM-CSF	IC and SC	Increased CFI
Zbinden *et al.*[41]	CAD	GM-CSF	SC	Increased CFI; prematurely terminated because two patients developed acute coronary syndrome
START [42]	PAD	GM-CSF	SC	No difference in maximal walking distance after 14 or 90 days
Subramaniyam *et al.* [43]	PAD	GM-CSF	SC	Non-significant increase in exercise capacity and improvement of endothelial function
Meier *et al.* [44]	CAD	G-CSF	SC	Increased CFI; reduced electrocardiographic signs of ischemia upon balloon coronary occlusion
Grossman *et al.* [45]	PAD	DEL-1 plasmid	IM	No change in exercise capacity at 30, 90 and 180 days
Belardinelli *et al.* [46]	CAD	Dipyridamole	Orally	Treatment improved coronary collateralization, especially when combined with exercise
TRAFFIC [47]	PAD	FGF-2	IA	Increase in peak walking time at 90 days; double dose no better than single dose
FIRST [49]	CAD	FGF-2	IC	No significant difference in exercise tolerance
Laham *et al*. [50]	CAD	FGF-2	Perivascular	Non-significant improvement of myocardial perfusion with high dose treatment
Grines *et al*. [48]	CAD	Adenoviral FGF-4	IC	Non-significant improvement of myocardial perfusion
VIVA [51]	CAD	$VEGF_{165}$	IC and IV	High dose treatment improved angina; no change in exercise capacity

CAD, coronary artery disease; CFI, collateral flow index; FGF, fibroblast growth factor; G-CSF; granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IA, intra-arterially; IC, intracoronary; IM, intramuscularly; IV, intravenously; PAD, peripheral arterial disease; SC, subcutaneously; VEGF, vascular endothelial growth factor.

Vascular endothelial growth factors (VEGF)

The most widely studied growth factor with regard to vascular growth is VEGF. This factor is known to stimulate angiogenesis i.e. capillary growth, by stimulating endothelial proliferation. However, some evidence is available for effects also on collateral artery growth. Positive results from small patient studies led to the initiation of a larger clinical study. However, the VIVA trial, using VEGF165 in 178 patients with CAD, did not show an effect on its primary endpoint, walking time [51].

From bedside to bench

Given the many dissimilarities between the experimental models and patients with CAD as discussed below, it has been suggested that the translation of knowledge from bench to bedside may not be the ideal approach for the design of new therapies for patients [35]. A better understanding of collateral artery growth in patients is necessary for the design of successful treatment strategies that are clinical applicable. Because of the large heterogeneity in the extent collateral artery growth upon arterial stenosis in patients, pilot studies have now focused on the identification of targets that relate to the capacity of the collateral circulation. Schirmer *et al*. dichotomized 50 patients with single vessel CAD according to their CFI_p and compared the transcriptome of the monocytes between these 2 groups. Interestingly, pathway analyses showed that genes related to the interferon-beta pathway, were highly expressed in the monocytes from patient with a low CFI_p. Moreover, the same research group demonstrated the inhibitory effects of interferon-beta on collateral artery growth in a murine hindlimb model, and thus, accomplished to validate their findings using a "bedside to bench" approach. Collectively, these results provide a novel mechanism involved in the regulation of arteriogenesis [54]. In another patient study, coronary collateral blood was samples from 60 patients with CAD to investigate cytokine levels in the collateral circulation. Interestingly, in the coronary collateral samples increased plasma levels were found for basic fibroblast growth factor, eotaxin, macrophage migration inflammatory factor, monocyte chemoattractant protein-1, and transforming growth factor-beta, while stem cell factor and stem cell growth factor-beta were decreased, as compared to coronary artery samples. These findings provide important insights into the importance of the several cytokines for collateral artery growth in CAD patients [55]. Based on the results from the patient studies, new therapeutic strategies have been designed to stimulate collateralization, which are currently under investigation for their feasibility.

Discussion and Conclusions

The importance of collateral artery growth has been recognized for many years. Well-developed collateral arteries are associated with reduced morbidity and mortality in patients with CAD. Likewise, therapeutic stimulation of collateralization seems an appealing concept to improve outcome. It had been estimated that 25-30 percent of the patients with CAD cannot be revascularized by PCI or CABG [34]. Hence, the stimulation of collateral artery growth, especially in this patient group, may be a valuable therapy. Catheter based hemodynamic measurements at the cardiac catheterization laboratory have opened new avenues to investigate the dynamic behavior of the coronary collateral circulation in patients with CAD. Additionally, these measurements provide an important tool to assess the efficacy of therapies, aiming at the improvement of collateral flow.

Physical exercise would theoretically be the ideal therapeutic option for the stimulation of arteriogenesis, since an increase in shear stress is the primary stimulus for remodeling of the arterioles. However, evidence in support of this hypothesis is still scarce, which may be related to use of inaccurate angiographic methods, insufficient patient numbers, or change in coronary stenosis severities [56,57]. Future studies are warranted to further evaluate the potential of therapies that increase shear stress. In the last decades, the exploration of collateral artery growth in experimental models has resulted in the identification of several growth factors involved in this process. However, the results of randomized clinical trials testing the effect of these growth factors on collateral artery growth have, in general, been disappointing. One should take into consideration several matters that may explain this controversy. For instance, the methods used in experimental models to assess collateral flow, such as laser-Doppler perfusion imaging, and microsphere perfusion under conditions of maximal vasodilation, are not feasible in patients. It is likely that the use of endpoints such as walking time or angina pectoris for the assessment of therapy in the patient trials are too crude to discriminate a subtle change in collateral flow over time. In addition, the angiographically based Rentrop-score does not accurately assess collateral flow, while advanced intra-coronary hemodynamic measurements for accurate assessment of collateral flow have been employed in only few studies. It should be further noted that many growth factors are also involved in the formation of small capillaries. Therefore, these growth factors may not have augmented the growth of collateral arteries, but only the development of small capillaries. In patients

with CAD, large collateral arteries are likely to be much more effective in the restoration of myocardial perfusion than small capillaries [58]. Another problem is treatment strategy. While several treatment strategies have been thoroughly investigated in experimental models, there are many uncertainties about optimal timing, dosage, delivery strategy and the need for multiple growth factors in patients. Finally, there are many genomic and biological dissimilarities between mice and man. An experimental model mimicking elderly patients with CAD and comorbidity such as diabetes mellitus and dyslipidemia, does not exist. This may explain why attempts to translate experimental findings into clinical practice have been unsuccessful, so far. Further research should focus on the molecular and cellular mechanisms involved in collateral artery growth in man, to develop new strategies to stimulate this process. Since large heterogeneity exists in the collateral response amongst patients with CAD, comparative studies of patients with either a reduced collateral flow or enhanced collateral flow may provide important insights into the background mechanisms involved in this process [54]. Integration of this knowledge in the design of future clinical trials may lead to the clinical implementation of therapeutic augmentation of collateral artery growth.

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Chapter 12. Stimulation of Arteriogenesis via External Counterpulsation - State of the Art and Clinical Perspective

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Abstract: Novel data has provided evidence for new approaches to the treatment of stable coronary artery disease. In a recent clinical trial it was demonstrated that the non-invasive promotion of coronary collateral artery growth is possible in patients with coronary artery disease. The technique of external counterpulsation (ECP), an art of "passive training", was used to increase physical forces that stimulate arteriogenesis. The Arteriogenesis Network 2 Trial (ArtNet 2) provided the first data for direct stimulation of coronary arteriogenesis by using invasive intracoronary pressure measurements to evaluate changes of the collateral blood flow after the ECP therapy. This chapter presents the main principles and effects of the ECP therapy in patients with cardiovascular disease. The data form the ArtNet 2 trial will be discussed in this chapter in detail.

Introduction to the External Counterpulsation

The term external counterpulsation refers to a non-invasive device used to achieve a diastolic augmentation analogue to that of the intra-aortic balloon counterpulsation. External counterpulsation was initially developed to help patients with acute myocardial infarction and heart failure as a non-invasive alternative to the intra-aortic balloon (IABP) [1-3]. The first experiments and clinical trials with external counterpulsation took place about 40 years ago, investigating the acute hemodynamic effects on the left ventricular workload and the coronary perfusion [3-5]. However, the first hydraulic device of counterpulsation proved to be less effective than the IABP for the treatment of cardiogenic shock [6]. An alternative non-invasive counterpulsation device based on air-filled cuffs came into use in the late 1970s [7]. The currently used counterpulsation systems are based on the latter technique.

ECP is nowadays indicated in the treatment of the refractory angina pectoris as suggested by the guidelines [8]. Other approved indications for use include unstable angina, congestive heart failure, acute myocardial infarction and cardiogenic shock [9]. The clinical benefits for patients with CAD include diminution of anginal symptoms, reduced uptake of nitroglycerine, improvement of the exercise tolerance and quality of life [10]. Three of four patients with refractory angina experience an improvement of at least one class in the classification of the Canadian Cardiovascular Society of Cardiology (CCS) and 38% of them of at least two classes immediately after the therapy. For most of the patients the benefits can be sustained up to three years after completion of the therapy [11]. The exercise capacity is also improved in the majority of patients [12-14]. Recent studies have demonstrated the safety and effectiveness of the method in patients with mild to moderate heart failure. In a clinical trial, exercise tolerance and functional class in classification of the New York Heart Association (NYHA) were improved after the therapy [15]. A retrospective analysis in patients with refractory angina demonstrated a significant reduction of the systolic pressure after a course of ECP [16]. The therapy may also be effective in other conditions such as hepatorenal syndrome [17], restless leg syndrome [18], erectile dysfunction [19], tinnitus [20] and, as has been shown recently, ischemic stroke [21].

Technical characteristics of ECP

The external counterpulsation therapy consists of electrocardiographic-triggered compressions of the lower extremities induced in the early diastolic phase followed by decompressions at the systolic phase of the cardiac cycle. Three pairs of cuffs, applied on the calves, lower and upper thighs, are inflated from distal to proximal during early diastole. The compressions result in augmented diastolic pressure and flow as well as in increased venous return. The cuffs are then rapidly deflated at the onset of systole leading to reduced vascular

resistance. The sequence of inflation and deflation during the therapy leads to increased diastolic pressure (diastolic augmentation) and decreased systolic pressure (systolic unloading – Fig. **1**). Increased perfusion of the brain, liver, kidneys and myocardium during the therapy was demonstrated by duplexsonography and transesophageal echocardiography [22]. The synchronization of the compressions with the cardiac cycle takes place automatically through a 3-canal ECG. The inflation-deflation activity is monitored by a finger plethysmography displaying respectively the systolic and diastolic amplitudes which are useful for the optimal adjustment of the therapy. Whereas the principal operation of ECP is automatic, the operator can optimize the hemodynamic effect by modifying the times of inflation and deflation of the cuffs. This adjustment is based on the achieved diastolic and systolic curves which are depicted continuously on the monitor of the device. From these curves the D/S ratio is automatically calculated. Previous trials have showed that an increase of the ratio during the treatment period is related to a better effect and outcome of the therapy [23]. Patients with the greatest increase of the ratio have the greatest reduction in angina class [24]. However, other trials suggest that the therapy is effective independent of an improvement of the ratio [25]. A maximal hemodynamic effect is achieved by an index of 1.5 or greater [26].

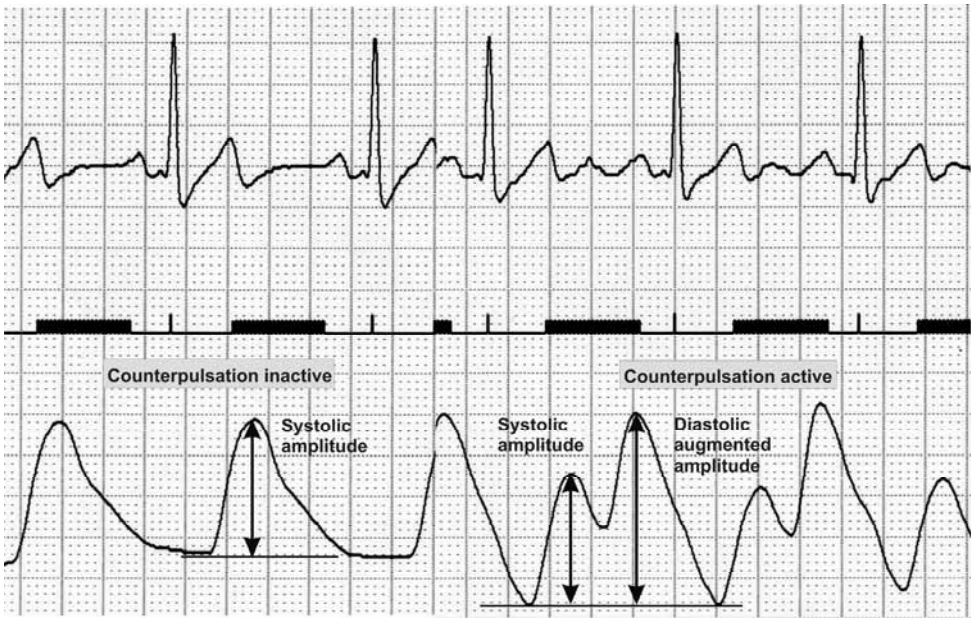

Figure 1: Finger plethysmography for the calculation of the D/S ratio and adjustment of the therapy. Links, before activation of ECP only a systolic wave can be seen. Right, after activation of ECP a second diastolic wave is produced.

D/S ratio= diastolic augmentation amplitude /systolic augmentation amplitude.

A typical course of ECP includes 35 outpatient treatments administered as 1-hour daily sessions over 7 weeks. This standard duration of counterpulsation treatment is based on empiric data derived from studies in China and has been proposed as the optimal course based on data from the international EECP® Patient Registry [27].

Mechanism of action

Though the technical principle of external counterpulsation is simple, the physiological response, especially the hemodynamic effects, is complex and the mechanism of action seems to be multilateral. Four main hypotheses are so far proposed to explain the clinical effects of ECP: i.) adaptive proliferation of coronary collateral arteries (arteriogenesis), ii) improvement of endothelial function, iii) improvement of left ventricular function and iv) decrease in peripheral resistances.

The first two hypotheses share a biomechanical effect of ECP: the increase of shear stress. Michaels and colleagues investigated by intracoronary pressure measurements and intracoronary Doppler flow the acute effect of ECP on cardiac function and circulation. They showed that during ECP intracoronary peak diastolic pressure was increased by 93% while peak systolic pressure was reduced by 15%. The peak diastolic coronary flow velocity was increased by 109% suggesting an improvement in coronary blood velocity [29]. In another study an increase of the blood flow volume in the brachial artery was demonstrated while the diameter of the brachial artery remained unchanged during counterpulsation [30].

The fluid shear stress (τ) is proportional to the blood flow (Q), the blood viscosity (η) and inversely related to the radius (R) of a vessel upon the equation $\tau = 4\eta Q/R^3\pi$. Taking into account the latter data, it is suggested that during ECP the shear stress in the vascular system is increased. Elevated levels of shear stress are crucial for the maintenance and improvement of endothelial function as well as for arteriogenesis [31,32]. The hypothesis of arteriogenesis will be discussed later in detail.

The hypothesis of improvement of the endothelial function is supported by clinical and experimental data. It is not only the increased shear stress but also the increased number of arterial pulsations per cardiac cycle during the counterpulsation therapy which exerts beneficial effects to the endothelium [33]. During the therapy a second pulsation occurs during diastole for each heart beat. By performing functional tests for the assessment of the peripheral endothelial function such as brachial artery flow-mediated dilation (FMD) or reactive hyperemia-peripheral arterial tonometry (RH-PAT) an improvement of the endothelium in response to ECP has been shown [34,35]. Circulating levels of important mediators of the endothelial function (cGMP and nitric oxide) are also elevated after ECP suggesting a positive effect of the therapy on the endothelial function [36,37]. It has also been reported that ECP reduces the circulating levels of inflammatory cytokines. Casey and colleagues demonstrated a reduction of the levels of the tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1) and vascular adhesion molecule-1 (VSCM-1) after ECP. Furthermore, stabilization of the endothelium, an effect similar to exercise training, was demonstrated in hypercholesterolemic pigs [38].

Other investigators showed reduced arterial stiffness post-ECP indicating peripheral vasodilatation and decrease of the peripheral resistances [39]. The increased artery stiffness is furthermore associated with reduced coronary blood flow possibly due to increased left ventricular workload and reduced coronary artery diastolic filling [40]. Briefly, ECP improves the peripheral endothelial function and reduces the peripheral resistances whereas no data exists about similar effects on the coronary endothelium.

The fourth hypothesis, supporting an improved left ventricular function, remains to the date in question. On the one hand, it has been shown that the left ventricular end-diastolic volume is decreased after the therapy and the left ventricular ejection fraction (LVEF), if abnormal at baseline, is increased after ECP [41]. Other investigators demonstrated that a marker of the left ventricular filling, the lung/heart ratio was reduced after ECP [42]. The decrease of the lung/heart ratio indicates a decrease of the LVEDP and left ventricular end diastolic volume [43,44]. A significant reduction of the atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) is also demonstrated in response to a course of ECP [45]. However, in a recently published study no improvement of any index of the left ventricular systolic and diastolic function was seen [46].

Arteriogenesis as possible mechanism of action

The hypothesis of arteriogenesis goes back to the 1970s. Jacobey and Rosenzweig examined post-mortem angiograms of dogs which were initially submitted to an acute or chronic myocardial infarction and consequently treated with counterpulsation therapy. They showed enhanced epicardial and sub-endocardial collateralization and reduced infarct size in dogs treated with counterpulsation compared to control dogs [47,48]. In a recent study, canines with myocardial ischemia were treated with an external counterpulsation model similar to that which is currently used in humans. The animals were submitted to occlusion of a coronary artery before being randomized in the active ECP group or the control group. After 6 weeks of counterpulsation, the perfusion defects in the initial ischemic infarcted areas were attenuated in the active group [49]. The improvement of the perfusion only in the ischemic areas suggests an angiogenic effect of the method.

Indirect evidence for the arteriogenic effect of ECP in humans comes from several studies having demonstrated improved myocardial perfusion after ECP. Masuda et al. [45] conducted a prospective study of 11 patients with at least one coronary stenosis (> 90%) before and after treatment with ECP. Myocardial perfusion, assessed by dipyrimadole ^{13}N-ammonia positron emission tomography (PET) increased after ECP suggesting an arteriogenic effect of the therapy [45]. Improved myocardial perfusion by myocardial scintigraphy and reduction of wall abnormalities assessed by stress echocardiography are evidenced by many clinical trials [13,14,50-55]. Most of the studies demonstrated that the reduced myocardial ischemia correlates with an improvement of the anginal symptoms suggesting recruitment and proliferation of collaterals as the most possible mechanism. However, two other studies showed no effect of ECP on myocardial perfusion despite a clinical improvement of the patients which was attributed by the authors as being a peripheral effect of the therapy [12,56]. The growth of collateral arteries distal to a stenosis leads to improvement of the regional perfusion at rest which increases further during a stress test when recruitment of collaterals occurs under myocardial ischemia. However, an improvement of the coronary endothelial function can also be detected in an imaging test as an improvement of myocardial blood flow at rest or under physical exercise. It is therefore obvious that both improvement of the endothelial function or the collateral growth after ECP could be detected as improved myocardial perfusion in the imaging tests of the previous studies. Furthermore, in the majority of the trials physical exercise tests (e.g., treadmill SPECT) were used to evaluate the effect of EECP on myocardial blood flow. By using these methods a possible contribution of the coronary endothelium to the coronary blood flow cannot be excluded. It is known that an impaired coronary endothelium may partly contribute to the perfusion defects demonstrated by SPECT [57,58] or inversely the improvement of the myocardial ischemia after ECP may be partly attributed to improved coronary endothelial function. Furthermore, these tests were mostly performed at the same levels of exercise (same double product) before and after ECP. In this case reduced myocardial oxygen demand due to a peripheral training effect and lowered peripheral vascular resistance supports the fourth hypothesis (peripheral training effect) as a possible mechanism for the attenuation of perfusion defects.

Only one study, performed from Urano and colleagues [13], had till recently used an invasive but unfortunately inaccurate method (Rentrop score) to assess a possible arteriogenic effect of ECP. In this study SPECT and coronary angiography with assessment of the Rentrop score were performed before and after ECP in patients with at least one residual coronary stenosis. The reduction of the perfusion defects in SPECT was not accompanied by a significant increase of the Rentrop score but it was related to a reduction of the left ventricular end-diastolic pressure [13].

Direct evidence for arteriogenic effect: the Art.Net. 2 trial

1. Hypothesis and design

The Arteriogenesis Network performed between 2007 and 2008 a clinical trial in patients with stable coronary artery disease to provide clinical data to examine whether arteriogenesis is the main mechanism underlying the beneficial effects of ECP. Other aims of the trial were to

investigate if myocardial blood flow improves after ECP and if this improvement correlates with the clinical response to the therapy.

By using the gold standard invasive method to detect myocardial collateral-dependent blood flow, the CFIp (pressure-derived Collateral Flow Index), we demonstrated a direct therapeutic effect of ECP on coronary collaterals in patients with stable angina pectoris and significant coronary stenosis.

The detailed design of the trial is published elsewhere [59]. Briefly, 23 patients met the following major inclusion criteria: (1) angiographically stenosis (>70%) of one major coronary artery diagnosed by visual assessment within the last 2 months; (2) no previous infarction in the myocardial region of interest (ROI) for the assessment of collateral blood flow; (3) positive non-invasive ischemic testing for the ROI and fractional flow reserve (FFR) < 0.8 and were included in the trial. Included study participants were randomized in a 2:1 manner to the ECP and the control group. Patients in the ECP-group received 35 hours of treatment over 7 weeks (5h/week) with an EECP-TS3 device (Vasomedical Inc., NY). Clinical symptoms, status and the diastolic-to-systolic ratio (D/S) were registered at each treatment session. ECP was performed by applying cuff pressures between 200-240mmHg to achieve a target D/S ratio > 1.0. To compensate for the non-therapy-related effect of ECP, the control-group had over 7 weeks 5 times/week a walk-in appointment (for non-study-related diagnostics, nutrition-counseling and physician's appointment).

The primary endpoints of the trial, the collateral flow index and fractional flow reserve, were estimated invasively during cardiac catheterization procedures before and after the period of 7 weeks. FFR was calculated as $FFR=P_d - P_v / P_a – P_v$ [60] and was used as a major inclusion criterion but also as endpoint to assess effects of ECP on the myocardial blood flow. The CFIp was estimated based on the next equation: $CFIp=(P_w-P_v)/(P_a-P_v)$ [61]. So, all patients twice underwent a cardiac catheterization examination with invasive coronary pressure measurements. No percutaneous coronary intervention was performed during the first diagnostic invasive procedure. During the invasive follow-up at week 8 and after having assessed the study-related hemodynamic measurements revascularization was performed or not based on the FFR value and according to the guidelines [19].

To assess symptoms standardized questionnaires on the CCS and the NYHA functional class were used. Within 3 weeks prior to the invasive diagnostics oral antihypertensive medication was adjusted to meet the guideline recommendations [62]. Baseline medication thereafter remained unchanged. Throughout the study patients were instructed not to change their daily activity.

2. Results of the Art.Net 2 trial

In the ECP-group the CFIp increased from 0.08±0.01 to 0.15±0.02 (no units, $p<0.001$), whereas the control group showed no variance of the index (0.15±0.03 to 0.14±0.02, $p=0.7$) demonstrating growth of collateral arteries in patients treated with ECP. In accordance to the CFIp, the FFR index increased in the ECP group from 0.68±0.03 to 0.79±0.03 (no units, $p=0.001$) but not in the control group (0.68±0.06 to 0.70±0.05, $p=0.4$). In the ECP group after treatment a significant reduction of the CCS classification was achieved ($p=0.008$) whereas in the control group no change ($p=0.25$) was observed. The severity of dyspnea (NYHA scale) was reduced after ECP ($p<0.001$) but not within the control ($p=0.28$). At the end of the therapy 81% of the ECP patients were free of angina pectoris (CCS=0) compared to 56% at baseline. In the control group at baseline and after 7 weeks only one patient (14.3%) was free of angina. No patient treated with ECP had an increase in angina class or remained in CCS > II after the therapy. The detailed results of the trial are presented elsewhere [63].

3. Stimulation of arteriogenesis

Important hallmarks of arteriogenesis are enhanced levels of shear stress across recruited collateral pathways. Experimentally a therapeutic increase in shear stress may be achieved via artificial stenosis, ligation or arterio-venous shunting distal the site of occlusion/stenosis [65].

ECP increases arterial shear stress even in the absence of a stenosis [66]. In humans, it is proposed as an elegant method to non-invasively enhance shear stress in the arterial system (within the cardiac diastole without increasing heart rate) [28,67]. In this proof-of-concept trial we evaluated whether counterpulsation leads to a significant change in CFIp and FFR as compared to the natural time course under optimal medical treatment.

The primary hypothesis of the Art.Net.-2 trial was to investigate the direct effects of counterpulsation on collateral function in patients with stable angina pectoris using the CFIp. The latter is currently the gold standard for the assessment of the collateral circulation. CFIp expresses the maintained collateral blood flow during coronary occlusion relative to normal antegrade flow during vessel patency [61]. In contrast to several other trials – which detected CFIp either at single time points or performed CFIp measurement within the PCI procedure – the ArtNet2 trial assessed CFIp at two longitudinal time points: baseline and follow up after 7 weeks. We observed a significant increase of collateral blood flow after ECP treatment, whereas in the control group no change was observed. At the end of the therapy course 6/16 patients in the ECP group versus 1/7 patients in the control group were deferred from angioplasty. In summary, the significant increase in CFIp is supported by a second functional endpoint, the FFR. The concurrent improvement of the CFIp and FFR in angiographically unchanged coronary arteries is evidence that the collateral arteries contribute to the improved myocardial blood flow which leads to the clinical improvement of the patient. The FFR takes into account the contribution of collaterals as long as the distal coronary pressure during maximal hyperemia reflects both antegrade and retrograde flow [68]. Thus, pressure-derived myocardial blood flow reserve improved upon ECP treatment significantly, reflecting the improvement of myocardial blood flow in the area at risk.

In the current trial the reduction in angina seen in all of the pre,ECP symptomatic patients was related to an improvement of the coronary functional status; suggesting that the amelioration of angina is considerably depending on the improvement of myocardial blood flow. However, due to the small number of symptomatic patients this data has to be further investigated in future trials. Since all patients, including those who where free of angina at baseline, had an improvement of effort-related dyspnea, the latter beneficial effect of ECP is likely to depend on synergistic effects; including improved diastolic function, increased exercise capacity and decreased peripheral vasculature resistance [35,69,70].

It is known that the growth of collateral arteries in humans, in contrast to those in animals, is a process which takes place during weeks to months [71]. In this trial the significant decrease of CCS and NYHA class was obtained in the second half of the therapy, suggesting that the commonly used ECP protocol of about > 30 hours of therapy is appropriate for an adequate clinical response.

The results of the Art.Net 2 trial were recently verified by another clinical study using CFIp and FFR as endpoints [72]. In the latter trial a very similar improvement of the CFIp of 0.075 was demonstrated only in the ECP group. In the sham group, treated with ECP at cuff pressure of 80mmHg, no change of the CFIp or the FFR was seen. Furthermore, the improvement of the CFIp was correlated with increased peripheral flow-mediated dilation (FMD) supporting the hypothesis that vasodilatation is important in the process of arteriogenesis.

Conclusions

The external counterpulsation seems to be a safe, non-invasive method to stimulate arteriogenesis in patients with stable angina pectoris. Based on diastolic augmentation and consequently enhanced shear forces, this concept of “passive coronary training” may serve as a valuable complementary treatment strategy to promote myocardial collateral growth in patients beyond active training or in patients who are not able to perform regular cardiovascular training. The current data from the two independently designed and performed trials mentioned above indicate a clear arteriogenic effect of ECP. As long as ECP is a therapy with almost no side effects when applied in an appropriately selected population, a supplementary use of the therapy beyond standard medication could be feasible for a large number of patients with stable CAD. However, the therapy is currently not broadly used basically due to the time needed to complete a course (7 weeks) and the relative high costs,

not covered in many countries by the insurance. Further pitfalls of current ECP treatment forms include the relative high noise during treatment and the unspecific approach (non-personalized treatment).

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Chapter 13. Future Perspectives

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Formation of the collateral arterial network during embryogenesis, the genetic and environmental factors influencing native collateral artery circulation as well as local and systemic mechanisms modulating collateral artery growth in the adult are research topics, currently receiving much attention. Although in the early stages, we are beginning more and more to comprehend the complex regulation involved.

Virtually every organ of the body is equipped with an arteriolar network; however, the number of arteriolar anastomoses seems to vary widely between individuals. Large variation in clinical outcomes of patients with vascular occlusive diseases might reflect differences in the capacity of remodeling, although only to the extent of pre-existing collaterals. Since a well formed arteriolar network is the pre-requisite for life and tissue saving arteriogenesis during vascular occlusive diseases a lot of emphasis is being put on the identification of the responsible genetic loci and genetic programs controlling the number, size and maintenance of arteriolar anastomoses during embryonic development as well as in postnatal development.

Until recently, most studies focusing on vascular development, i.e. angiogenesis, investigated embryonic principles in the adult organism under patho-physiological conditions such as ischemia or during tumor growth. However, angiogenesis and arteriogenesis, as well as collaterogenesis (formation of collaterals), differ considerably. Alternative approaches aiming at understanding the molecular mechanisms governing the collateral formation and branching pattern in the embryo had just begun.

Factors like VEGF-A, VEGFR-1, Angiopoietin-2, PDGF-B and CLIC 4 have been identified to be important in formation and maturation of the proto-collateral plexus. The Notch signaling pathway has been identified to be essential for arterial development and growth, both during development and after birth. Furthermore, it is clear that hemodynamic forces and increased blood flow conduct the orchestrated action. Although still a hypothesis, it is becoming more and more evident that (neo-)collaterals form from fusion of capillary-like branches from established arterioles rather than de novo. The process of “upgrading” capillaries into small arterioles is likely to be a very slow process and it is uncertain whether it has any physiological relevance during the early phase after arterial occlusion. However, experimental evidence has clearly shown a positive association between the number of preexisting arterioles and the outcome of vascular obstructions. Patients at risk with sparse collaterals might benefit from collateralogenic therapies before an obstructive vascular event occurs, profiting from the existence of collaterals and subsequent therapies augmenting collateral artery growth.

Investigations on the molecular mechanisms governing the formation of collaterals in the embryo are designed to provide clues regarding the signaling molecules responsible for the formation of neocollaterals in the adult. It is, therefore, a future challenge to identify the full repertoire of genetic mechanisms and molecules involved in the formation of proper collaterals being eventually able to offer effective therapeutic approaches.

Another challenging task will be to unravel the process of pruning. Again, studies of the embryonic and fetal, but also adult organism, might further the knowledge on maintenance of collaterals. In particular the embryonic and fetal vasculature is highly plastic and hemodynamic forces, but also environmental influences, are likely to determine the fate of collaterals either in the direction to be sustained or pruned. However, the exact molecular mechanisms remain to be elucidated in order to be able to prevent pruning and warrant abundant collateralization.

Although arteriogenesis per se is defined as the growth of pre-existing collaterals, it is of similar importance to define the mechanisms of collaterogenesis. Epigenetic regulations are very likely to be involved in both processes. Epigenetics defines the heritable changes in

genome function that occur without a change in DNA sequence. It involves DNA methylation but also histone modifications regulating the "on"/"off" state of gene expression. Currently, little data on epigenetics and collaterogenesis or arteriogenesis are available. Nevertheless it is clear that epigenetic regulations play a major role in both processes. Certain genes, such as eNOS, FGF-2, PDGF, SRF, that are known to have mechanistic functions in collateral formation as well as growth, fall under epigenetic regulation. To define all the genes involved in collaterogenesis and arteriogenesis is certainly a future task; however, it will be a greater challenge to identify the elicitors of epigenetic modifications. The fundamental question is, what induces the epigenetic regulation of a single gene (or multiple genes) during various life stages, e.g. during pregnancy, during normal life and in an aged person? What are the environmental influences, what are the effects of nutrition, and finally - and even more difficult to define - what influence does the status of an individual's psychic well-being have?

Whereas collaterogenesis is a relatively new discipline, in-depth investigations on arteriogenesis started about 15 years ago. Accordingly, our knowledge on signal transduction cascades after activation of the endothelium, beginning with the activation of the Rho-kinase pathway and resulting in the downstream activation of the cell cycle regulator Egr-1, as well as the influences of bone-marrow derived cells (monocytes, T-cells), is much larger in arteriogenesis. However, our understanding how the mechanical force, the increased fluid shear stress that is the trigger of arteriogenesis, is translated and induces the diverse biological reactions which lead to vascular cell proliferation and outward remodeling, is still poor. The initial understanding of the functional role of ions (in particular Ca^{2+}) as well as ion channels (i.e. TRPV4), which began when the book "Arteriogenesis" was published by Wolfgang Schaper and Jutta Schaper in 2004, has now deepened significantly. However, the mechanistic function of NO and NO-synthases is less clear. Nitric oxide is anti-proliferative for SMCs and counter-acts the adhesion of leukocytes to the endothelium – two processes of fundamental importance for arteriogenesis. Yet NO, (or NO-synthases), are indispensable for proper collateral artery growth. Nitric oxide synthases, eNOS, iNOS and nNOS all seem to have elementary roles in this process, although they might substitute each other at least in part. And what about NO-donors? Administration of NO-donors like detaNONOate was shown to dramatically promote collateral artery growth. However, is it really NO? All forms of nitrogen can be changed into each other. Is nitrogen itself the important element? And what about ROS like H_2O_2? What about uncoupling eNOS resulting in increased H_2O_2 production? Or is it H_2O_2 that increases NO through eNOS activation and may also induce an NO-insensitive state? The correct redox balance seems to play the utmost fundamental role, not only for vascular cell proliferation, but also for the translation of the mechanical force (fluid shear stress) into the certain biological reactions. A thorough understanding of the function of the redox state will be one of the outstanding tasks for the future.

Finally, it will be a major challenge to transfer the knowledge from bench to bedside. Currently, approaches to promote arteriogenesis in the clinic have failed – except external counterpulsation. This is likely to be based on the fact the collateral artery growth is a multi-factorial process; almost excluding that on single substance may do the job. Thymosin β4 might present a promising exception. This peptide is not only of particular interest because it promotes arteriogenesis efficiently – at least in experimental settings – but also due to its anti-inflammatory feature *not* promoting atherosclerosis in parallel. Local drug delivery systems currently developed are promising tools finally rendering it possible to achieve a targeted and sustained drug delivery enabling effective arteriogenic therapies without severe systemic side effects.

Still a lot of work remains to be done. Hopefully, we will make it, like the bird will catch the (yet for us invisible) fish. However, we should not forget that the stakes are high: clinicians are very successful with their interventional techniques to open occluded arteries and we have to be better than them.


by S. Leese

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Wolfgang Schaper studied medicine and obtained his MD by thesis in 1958 at the Martin Luther University of Halle/Germany. He started his scientific career as a pharmacologist in Belgium and obtained his PhD at the University of Leuwen/Belgium in 1967 with a thesis on DNA and RNA synthesis in developing collaterals of the heart. Together with his wife Jutta Schaper he studied the ultrastructure of said vessels and in 1975, they conjointly discovered the role of monocytes. At the Max Planck Institute in Bad Nauheim (1972-2004), Wolfgang Schaper focussed his interest on the physical (fluid shear stress), cellular and molecular pathways of developing collaterals with the aim to search for a chemical that stimulates their growth. He is presently advisor of the Arteriogenesis Research Group at the Max Planck Institute in Bad Nauheim/Germany.

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