CONTENTS

Abbreviations and acronyms

3V-UTR	3V-untranslated region
5-FU	5-fluorouracil
ACA	Anterior cerebral artery
AP-1	activator protein 1
ATP	Adenosine Triphosphate
AV node	Atrioventricular node
CCR-2	CC chemokines receptor 2
CD	cluster of differentiation
CL	Cytoplasmic loop
CS	Conduction system
CT	Computed tomography
CWS	circumferential wall stress
CX	Connexin
EGFP	enhanced-green fluorescent protein
Egr-1	Early growth response 1
EL-1	Extra-cellular loops-1
EL-2	Extra-cellular loops-2
EPC	Endothelial progenitor cells
ER	Endoplasmic reticulum
FA	Femoral artery
FAO	Femoral artery occlusion
FGF-1	Fibroblast growth factor-1
FGF-2	Fibroblast growth factor-2
FSS	Fluid shear stress
GAPDH	glyceraldehyde phosphated dehydrogenase
GC muscle	Gastrocnemius muscle
GM-CSF	Granulocyte macrophage colony-stimulating factor
Gj	Gap junctions
Gja5	Gap junction alpha-5
Het	Heterozygous
ICAM-1	intracellular adhesion molecule-1
i.p.	Intraperitoneally
KO	Knockout
LDF	Laser Doppler Flow
Mac-1	Macrophage 1 antigen
MCA	Middle cerebral artery
MCP-1	Monocyte chemoattractant protein-1
MicroCT	Micro-computed tomography
MMP	matrixmetalloproteinase
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PComA	Posterior communicating artery
PDGF	Platelet-derived growth factor
PET	Positron Emission Computed Tomography
PlGF	placenta growth factor
RG	Right gastrocnemius
RGS-5	Regulators of G protein signaling-5

RNA	Ribonucleic acid
RPSI	Relative pulse slope index
ROI	Region Of Interest
RT-PCR	Reverse transcription polymerase chain reaction
SMCs	Smooth muscle cells
TIMP	tissue inhibitor of matrixmetalloproteinase
TNF-a	Tumor necrosis factor-a
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2
WT	Wild-type
ZO-1	Zona occludens-1
ZO-2	Zona occludens-2

CHAPTER ONE: INTRODUCTION

1. Arteriogenesis

1.1. Three different ways of neovascularization

Cardiovascular disorders are currently the leading cause of death globally. Although successful therapies exist to reduce plaque formation and restore blood flow in patients suffering from ischemic vascular diseases, there is still a significant portion of patients who do not benefit from these treatment options. For a long time, it has been known that patients suffering from coronary heart disease can recruit collateral vessels and thereby improve symptoms of myocardial ischemia[1]. Also, it is well established that an increased demand for oxygen, as occurs during exercise and placental development, can induce formation of new capillaries[2]. Thus, it seems that the body already possesses an "in-house" rescue system to increase blood flow in ischemic circumstances. Stimulation of this system, termed neovascularization, could be a promising new direction in treating cardiovascular diseases[3]. Neovascularization in humans can be brought about by three distinct mechanisms: vasculogenesis, angiogenesis, or arteriogenesis (depicted in **Fig. 1.1.**)[3].

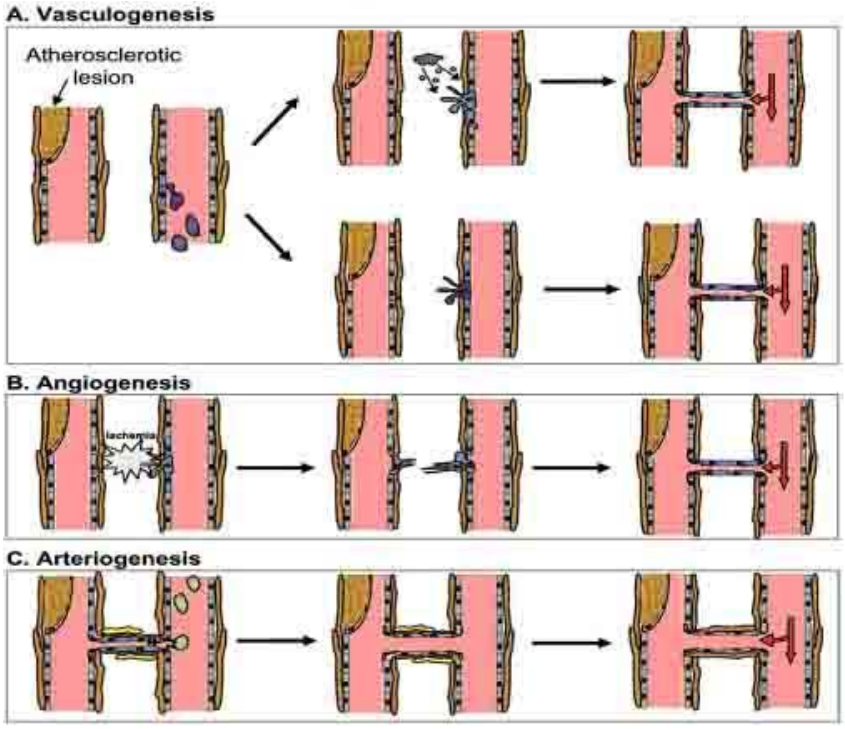
During embryonic development, blood vessels form de novo from angiogenic blood islands in a process termed vasculogenesis. This primary plexus extends by capillary sprouting and eventually remodels into a highly organized network of capillaries, arteries and veins[4]. The postnatal vascular system is critical for maintaining homeostasis and adapts readily to environmental cues and physiological or pathological conditions[5]. This adaptation comprises two different and characteristic responses, angiogenesis and arteriogenesis.

1.2. The definition of arteriogenesis

The term "arteriogenesis"—the development of large collateral arteries from pre-existing arteriolar anastomoses—was proposed in 1997 by W. Schaper, R. Chapuli-Munoz, and W. Risau[7] to discriminate between arteriogenesis and true angiogenesis. Arteriogenesis is defined as the enlargement of pre-existing collateral arteries and their remodelling into conductance vessels[8]. This process is driven by an

increased blood flow in collateral arteries leading to an increase in wall tension and fluid shear stress[9-11]. Specific arterial signaling pathways, angiogenic growth factors, as well as resident cells in the vessel wall and circulating cells participate in this complex biological process of luminal expansion and wall growth[12-17]. It is important to note that arteriogenesis is the key mechanism to enhance perfusion and is, thus, critical for the rescue of ischemic organs[18, 19].

Fig. 1.1.



[6] (Van Oostrom, M.C., et al., J Leukoc Biol, 2008；84(6): 1379-91)

Fig.1.1. Neovascularization can occur via vasculogenesis (**A**), angiogenesis (**B**), or arteriogenesis (**C**). (**A**) In vasculogenesis, circulating endothelial progenitor cells (EPC; purple) contribute to new blood vessel growth (capillaries). (**B**) During angiogenesis, endothelial cells are activated by ischemia and develop a lumen, thereby forming a new, small capillary vessel[3]. (**C**) In arteriogenesis, circulating leukocytes (green) are attracted to the activated endothelium. They assist in enlarging collateral anastomoses. Activated endothelial cells (blue), activated vascular smooth muscle cells (yellow)[3].

Normally, there is only a minimal net flow in these pre-existing connections. However, a sudden arterial occlusion or a slow progressing stenosis in the main artery can cause an increased pressure gradient in these small vessels to respond by actively proliferating and remodeling, which results in an increased lumen size and enhanced

perfusion to the ischemic tissue[20]. Hence, it seems that arteriogenesis is initiated differently and progresses differently from angiogenesis.

1.3. The process of arteriogenesis

Growth of collateral blood vessels (arteriogenesis) is potentially able to preserve structure and function of limbs and organs after occlusion of a major artery. The success of the remodeling process depends on the following conditions: (1) existence of an arteriolar network that connects the preocclusive with the postocclusive microcirculation; (2) activation of the arteriolar endothelium by elevated fluid shear stress; (3) invasion (but not incorporation) of bone marrow–derived cells; and (4) proliferation of endothelial and smooth muscle cells[9].

1.3.1. Arteriole

Arteriogenesis is the rapid proliferation of pre-existing collateral arteries. These vessels are microvascular, thin-walled conduits that are composed of an endothelial lining, an internal elastic lamina, and one or two layers of smooth muscle cells[21]. This view, that pre-existent arteriolar connections exist that enlarge by growth in response to arterial occlusion, still stands today[22-24]. The presence of these native collaterals, which may not be utilized to provide perfusion under normal conditions, varies widely among species and also within individuals. However, these vessels have the ability to dramatically increase the lumen by growth so as to provide enhanced perfusion to the jeopardized ischemic regions. In case of chronic or acute occlusion of a major artery, collateral arteries can relieve the ensuing harmful effects in many regions of the body (hindlimb, heart, brain and kidney). It is important to recognize that this process is not a passive dilatation but one of active proliferation and remodeling. Under normal flow conditions and depending on the pressure gradient between the interconnecting arterial networks there is only minimal net forward flow, but small amounts of flow may oscillate within the network.

Mature collateral vessels differ only in minor histological aspects from normal arteries of the conductance type: they are muscular and contain more collagen and exhibit transiently, during the growth process, a significant intima consisting of smooth muscle cells in the synthetic and proliferative phenotype. However, they differ markedly in their

anatomical appearance: they are sometimes excessively tortuous[13, 25]. In the re-entry region, they join up with the distal part of the occluded artery at nonphysiological angles, which adds to the resistance to flow. Collateral arteries can develop relatively quickly provided a pre-existent network of arterioles had existed before occlusion of the artery but they can also quickly regress when the occluded artery is opened up again[22]. This may also be the case when the subtended tissue had atrophied or is not used to full potential like in the peripheral circulation supplying the muscles of the leg. Most often, an occluded artery is not replaced by one single large collateral vessel but rather by several smaller ones. But this arrangement is inefficient because according to the Poiseuille's Law the energy losses created by the resistance of the contributing vessels are additive[9]. During the course of collateral artery development many of the smaller contributing vessels regress, whereas the larger ones increase in diameter and make the system more efficient. However, no ideal adaptation is reached. At optimal conditions (no tissue loss after arterial occlusion), collateral vessels recover only approximately 40% of the maximal conductance (flow at a given blood pressure at maximal vasodilatation). This was shown for the canine heart and for the peripheral circulation in pigs, rabbits, and mice[7, 26].

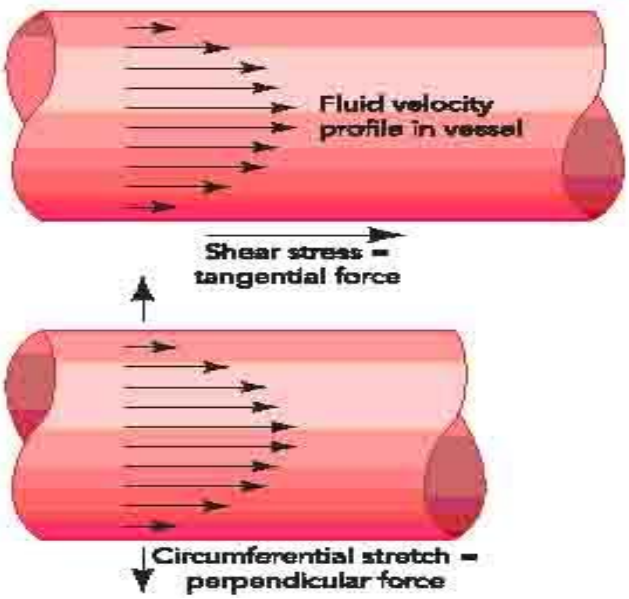
1.3.2. Fluid shear stress (FSS) as initial triggering

The initiation of blood flow creates mechanical forces on the developing endothelial cells. As a fluid flows through a tube, it exerts a force tangential to the tube, called shear stress, and another that is perpendicular to the tube wall and is caused by the pressure in the vessel, called circumferential stretch. (**Fig.1.2.**) Mature vessels can react to both these forces. Shear stress from blood flow has been shown to cause changes in morphology, cytoskeleton organization, ion channel activation, and gene expression within endothelial cells in vitro[27]. The collateral vessel wall is now exposed to various pronounced mechanical forces: increased blood flow directly augments FSS, i.e., the viscous drag that flowing blood exerts on the endothelial lining. Assuming Newtonian fluid dynamics, FSS can be estimated using the following equation:

$$\tau = \frac{4\eta Q}{\pi R^3}$$

The equation that already includes blood viscosity (η) and the internal radius of a vessel (R), demonstrates that increased blood flow (Q) will directly result in increased FSS (τ)[9]. Furthermore, the wall of the collateral arteriole is influenced by pressure-related forces like longitudinal-, circumferential-, and radial wall stresses.

Fig.1.2.



[28](Jones, E.A., et al, Physiology (Bethesda), 2006; 21: 388-95)

Fig.1.2. Forces created by flowing blood fluid flow within a tube creates two types of forces: shear stress, which is a force tangential to the vessel wall, and pressure, which creates a circumferential stretch perpendicular to the vessel wall. The velocity profile (shown by the arrows) within the vessel and the viscosity of the fluid determine the amount of shear stress to which the endothelial cells are exposed[28].

It is generally assumed that a physical stimulus starts the remodeling process whereby increased pressure leads to increased wall thickness and increased flow to increased arterial diameter. Pressure-dependent forces are by far the highest in magnitude and they affect both the endothelium as well as the muscular media. It is therefore logical to assume that these are important factors for remodeling[13, 25]. However, in collateral growth with its pressure gradient driven increase in flow, the much weaker FSS, which the viscous drag of flowing blood exerts on the endothelial

lining, is the determining force[11, 29-35]. The pressure derived pulsatile stretch is also discussed[25, 36, 37], and the transcription factor- activator protein 1 (AP-1) is the molecular transducer. However, AP-1 is also activated by FSS[38], Pulsatile stretch can only be tested acutely and in vitro with its inherent limits. Cultured endothelium under stretch alters translation and transcription of growth factors and changes the sensitivity to cytokines[11, 39, 40]. If pulsatile stretch is a molding force, it must be demonstrated that in collateral growth pulsatile stretch is higher than the physiological levels in normal small arteries. Furthermore, in arterial occlusion the intravascular pressure downstream from the occlusion is much lower than the systemic arterial pressure[8].

In the case of a sudden arterial occlusion or a slowly progressing stenosis, a steep pressure gradient develops along the shortest path within the interconnecting network that increases the blood flow velocity and FSS in these vessels. The effect of this sustained increase in shear is the upregulation of distinct processes in the collateral arteries. FSS with a range of 20 to 30 dynes/cm^2 is a weak force compared with circumferential wall stress (CWS), which is 10^6 times higher. FSS is proportional to the blood flow velocity and inversely related to the cube of the radius[41]. It is sensed by the endothelium, which, in response, changes the expression of growth factors, secretes nitric oxide (NO)[42, 43], prostacyclin, and probably other transmitters, and leads, with prolonged exposure, to positive arterial remodeling. However, even small increases in the radius of collateral arteries lead to a precipitous fall of the FSS because of the cubic relationship, and the FSS-related growth ends prematurely[20]. Furthermore, FSS is almost impossible to measure in small collaterals. Pipp and colleagues[44] demonstrated that sustained, elevated FSS in their arteriovenous shunt model further significantly increased the size of collaterals, thus establishing that FSS is a dominant morphogenic force in collateral growth.

Collaterals increase their diameter up to 20 times during arteriogenesis, which is possible through mitosis of vascular cells[45]. Given that the collateral vessels grow in length as well as in width, the expanding vessel arranges itself in loops and turns to accommodate the extra length. This gives the vessels a typical corkscrew pattern and causes energy loss[10]. This, together with the premature arrest of arteriogenic growth,

as a result of the drop in FSS in the growing collateral, is a reason that collateral arteries cannot completely compensate the conductance of the artery they have replaced. Initially, during arteriogenesis, several collateral vessels are recruited and proliferate. However, as it is hemodynamically more efficient for fewer, larger arteries to conduct the blood than a greater number of smaller arteries, the smaller vessels regress later on, and those with the higher shear forces continue growing[46].

FSS as a molding force was recognized over 100 years ago; the embryologist Thoma described the relationship between the diameter of an artery and its blood flow velocity[47]. Murray[48] proposed that the vascular system is optimally configured to minimize the amount of mechanical and metabolic work to provide adequate blood flow, and he predicted that FSS is constant throughout the vasculature and that blood flow through each vessel is proportional to that vessel's diameter cube. Any sustained deviation from that relationship initiates processes of either growth or atrophy. A sustained increase of fluid shear stress leads to activation of the endothelium.

1.3.3. Activation of the endothelium

It is currently not well enough known how the stimulus of increased shear stress is transmitted from the endothelial cell membrane to the nucleus, where it initiates the transcriptional activity of a number of genes, partially via a protein that binds to the shear stress responsive element that is present in the promotor of several genes (nitric oxide synthase (NOS), platelet-derived growth factor (PDGF), monocyte chemoattractant protein-1 (MCP-1))[49]. The first step in the activation of the endothelium is the opening of chloride channels that are also responsible for the volume control of endothelial cells. Characteristically stress-activated endothelium appears swollen in scanning electron microscopic images[50], adhesion molecules are upregulated[51], and the conditions are perfect for the adhesion and invasion of circulating cells.

Chronically increased shear stress activates endothelium in a morphologically visible way. It loses volume control and swells, because chloride channels change their open probability[52]. Inhibitors of the chloride channel also inhibit arteriogenesis. The location and nature of the mechanotransducer of shear stress are controversially

discussed[53], and protein kinases and stretch sensitive K-channels were studied[54]. We found that high shear stress in vitro causes a transient phosphorylation of focal adhesions, which could also act as mechanoreceptors[55]. By whatever way the mechanical force is transmitted from the deformed cell (membrane) to its nucleus, it will activate transcription factors, like early growth response 1 **(**egr-1**)** (upregulated during the early phases of arteriogenesis), that switch on gene expression, notably of chemokines like MCP-1 but also adhesion molecules like intracellular adhesion molecule-1 (ICAM-1), that are necessary for the docking of monocytes[56]. Shear stress is also known to release NO, but it is not known whether chronically increased shear stress will lead to chronically increased amounts of released NO. A lasting steep increase in shear stress leads only to a transient increase of egr-1, and this may also happen with the NO response[57]. The increased permeability of immature collaterals may have been caused by NO. However, expression studies on the RNA level have not shown any changes related to the early stages of collateral growth[20]. Immunofluorescence studies have shown the presence of PDGF antigen in neointima formation in canine collaterals, which supports findings by the Geary，R.L., et al[58]. showing that PDGF is increased under low-flow conditions that favor intima proliferation. The necessity of a cell-to cell transmitter (i.e., from endothelium to smooth muscle) is not very high, because the adhering monocyte assumes that function.

1.3.4. Circulating cells invade arterioles with activated endothelium

The upregulated expression of MCP-1 by endothelium attracts monocytes that adhere to and invade arteriolar collaterals. They in turn become activated, produce tumor necrosis factor-α (TNF-α) and attract more monocytes[21].

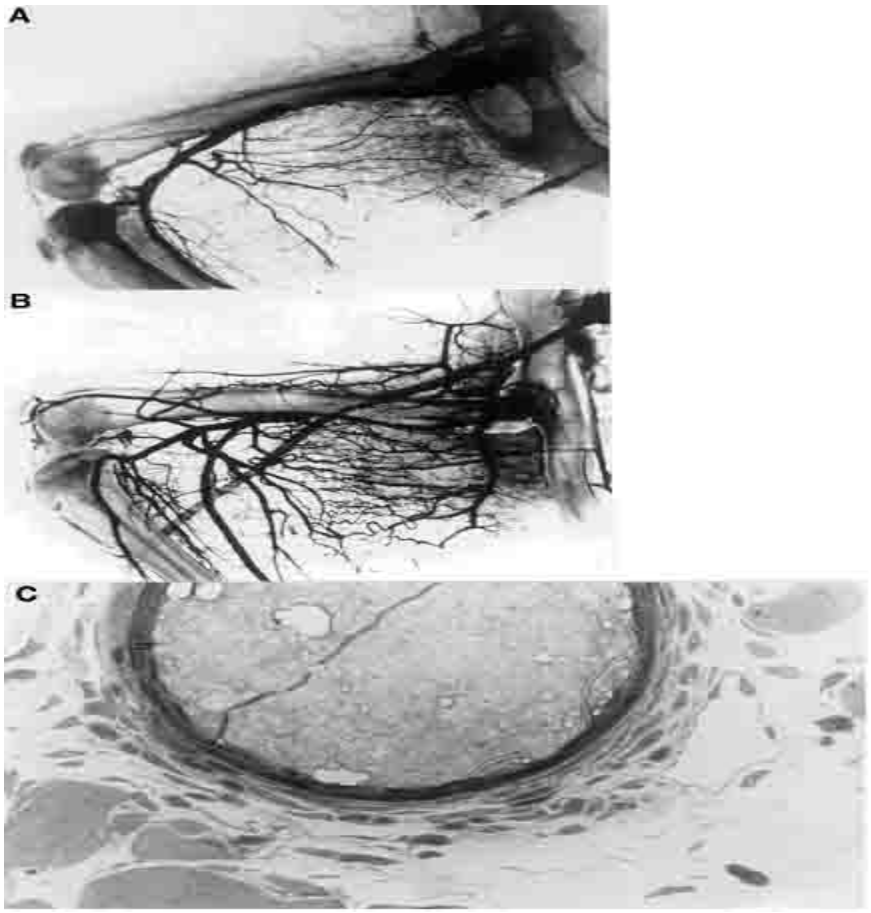
The endothelial lining of growing canine coronary collaterals is studded with monocytes that had attached, during phase 1 of arteriogenesis, to the now much rougher surface of the swollen endothelial cells that, activated by shear stress, upregulate the MCP-1 and adhesion molecules to which the macrophage 1 antigen (Mac-1) receptor of monocytes binds[55]. Infusion of soluble ICAM-1 binds to circulating monocytes and prevents their adhesion to transforming arterioles. The same results can be obtained with

intravenous infusion of anti–ICAM-1 antibodies that also prevent monocyte attachment. Targeted disruption of the MCP-1 receptor (CC chemokines receptor-2) (CCR-2) in mice prevented almost all collateral growth after femoral artery occlusion[59], but infusion of MCP-1 into the proximal stump of the occluded femoral artery led to increased monocyte influx and elicited a strong arteriogenic effect[60]. We also discovered that the weak arteriogenic effects of chronically infused vascular endothelial growth factor-A (VEGF-A) is caused by the monocyte attractant effect of VEGF that binds to the VEGF receptor 1, which is exclusively present on monocytes[61]. A similar effect was discovered with placenta growth factor (PlGF). The arteriogenesis-inhibiting effect of targeted disruption of PlGF in mice [62] could be lifted by bone marrow transplantation, i.e., an effect of monocytes[62, 63]. Because infusion of VEGF-E, which binds exclusively to VEGFR-2, did not influence arteriogenesis, we concluded that the effects of VEGF-A on arteriogenesis are caused by monocyte activation[64]. Intravenous infusion of liposome-packaged phosphonates (alendronate) destroyed all monocytes/macrophages for a period of ≈1 week. During this time, VEGF and PlGF infusions remained completely inactive, showing again the importance of monocytes in arteriogenesis[64]. Suppression of monocyte counts by treatment with 5-fluorouracil (5-FU) significantly delayed arteriogenesis, but the rebound effect after chemical bone marrow suppression had the opposite effect[20, 59].

Upregulation of survival factors for monocytes (granulocyte macrophage colony-stimulating factor **(**GM-CSF)) provides the environment for a stable function of monocytes (**Fig.1.3. C**). These in turn produce fairly large amounts of growth factors, including VEGF, colony stimulating factor, transforming growth factor-β, in particular, FGF-2[20]. The adhesion and invasion of monocytes and platelets (also potent producers of growth factors) is soon followed by the first wave of mitosis of the endothelial and smooth muscle cells. The cell invasion is most prominent in the intima, the initial entrance, but even more pronounced later in the adventitia, where they create an inflammatory environment that is later accompanied by T cells. One of the effects of the perivascular inflammation is that it creates the space (by forcing neighboring tissue cells into apoptosis) for the greatly expanding collateral vessel, which can increase its

diameter up to 20 times[21].

Fig.1.3.



[21] (Buschmann, I. and W. Schaper, News Physiol Sci, 1999; 14: 121-125)

Fig.1.3. A: angiography of the rabbit hindlimb (7-day phosphate-buffered saline infusion (PBS)) after femoral artery occlusion (FAO). B: angiography of the rabbit hindlimb [7-day MCP-1 infusion] after FAO. The number as well as the density of the collateral vessels increased significantly. Collateral arteries show a typical corkscrew pattern. C: histological section (midzone of the proliferating collateral artery) after 7 days of MCP-1 infusion. Several macrophages can be observed around the vessel[21].

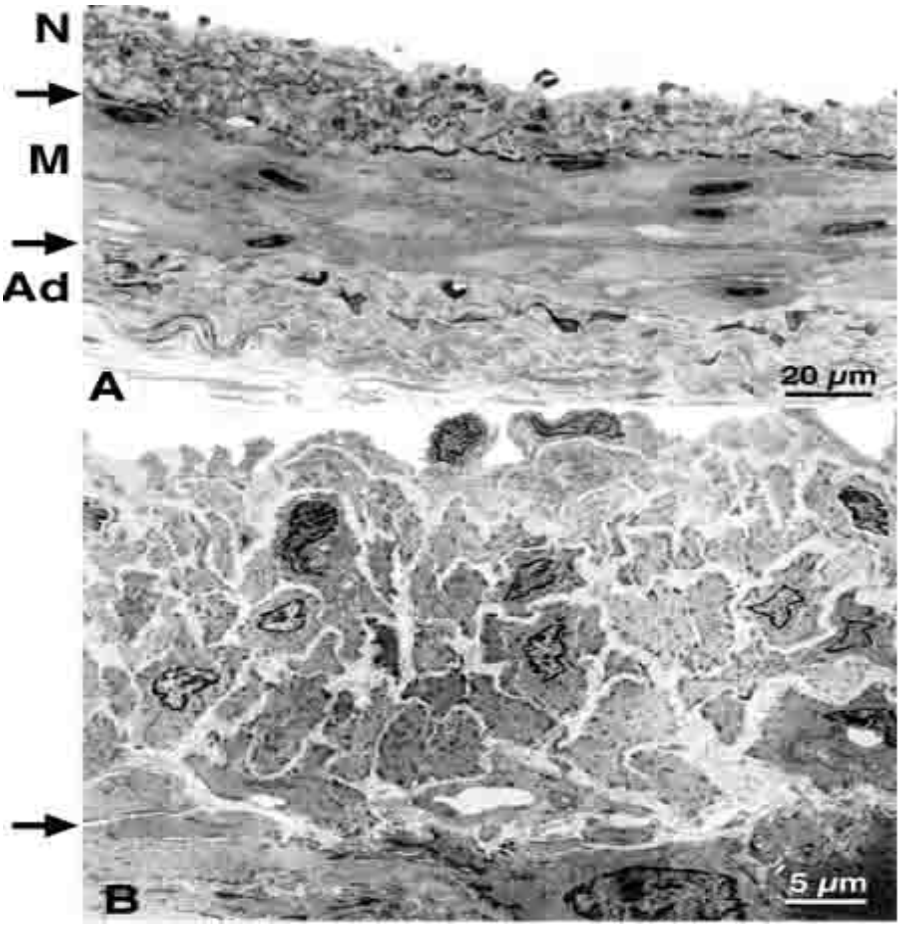
1.3.5. Remodeling

After the acute phase of arteriogenesis that is dominated by the inflammatory events, remodelling begins (phase 2 of arteriogenesis), i.e., the much slower consolidation of the arterial structure after the final diameter was almost reached. A new elastic lamina is synthesized by the SMCs, and the rebuilding of the media and the formation of an intima begins with the downregulation of the tissue inhibitor of matrixmetalloproteinases (TIMP and MMP)[65]. This is followed by an upregulation of

the expression and activity of the MMPs that digest the matrix and provide the space for new cells and enable SMCs to migrate toward the intima. Many SMCs of the old media undergo apoptosis and are replaced by new ones. Those that proliferate change their phenotype and lose most of their contractile material, which is replaced by endoplasmic reticulum (ER) and free ribosomes, an indication of their synthetic activity[18, 55]. The loss of the contractile phenotype is ascribed to the combined activities of protein kinase G, activin, and regulators of G protein signaling-5 (RGS-5). In addition to actin and myosin, desmin and calponin are downregulated and fibronectin is upregulated[66]. In general, protein synthesis in SMCs switches to an embryonic pattern. Because the thickening of the vessel wall occurs under markedly increased tangential wall stress, the intercellular connections and the communication between cells change. The remodelling process of large collaterals is finally characterized by the significant increase in length (tortuosity) and by the formation of a substantial intima (**Fig. 1.3.**)[21]. At very late stages, the intima disappears in mature collaterals, probably because the longitudinal muscle had assumed first a helical and later a circumferential orientation. In very small animals, like mice, neither intima formation nor pruning is observed, most probably because the increase in new tissue mass is so small that remodelling processes are not required[20]. However, already in the rabbit a sizeable intima is seen in hindlimb collaterals sometime after FAO. It is tempting to speculate that collateral arteries develop from the inside out using the intima as a platform; this is the incubator where the growth factors are produced, where the MMPs and other proteases are activated, and where the SMCs migrate to and then proliferate, thereby weakening the media from which they leave, producing the bulge of later tortuosity[20].

After months (6–12 months in the canine heart model) the new collateral artery is almost indistinguishable from a normal artery, except for a slightly higher collagen content between the smooth muscle layers. In particular, the prominent intima is no longer detectable[21].

Fig. 1.4.



[20](Schaper, W. and Scholz, D., Arterioscler Thromb Vasc Biol, 2003; 23(7): 1143-51)

Fig. 1.4. Typical histological/ultrastructural image of a collateral vessel wall 42 days after FAO (rabbit). **A**, interrupted thick black line (upper arrow) is the old lamina elastica interna. It marks the bottom of the neointima (N). The adventitia (Ad) containing collagen and the lamina elastica externa appear normal. Semithin section, stained with toluidine blue. **B**, the neointima is composed of SMCs, which are smaller than those in the media (M) and differently arranged but show the contractile phenotype. Ultrathin section, contrasted with uranil acetate and lead citrate[20].

1.3.6. Therapeutic arteriogenesis

In previous studies, Ito, W.D., et al., showed that chronic intra-arterial infusion of MCP-1 greatly increased the development of arterial collateral blood vessels (arteriogenesis) after FAO[7, 60]. These collaterals were more numerous on angiograms, and their ability to conduct blood had increased by six fold. (**Fig. 1.3.**, **A** and **B**) The histological appearance of these typical corkscrew vessels was that of muscular arteries[20]. In another study, Arras, M., et al.[14] injected a single dose of

lipopolysaccharide intravenously into New Zealand white rabbits 3 days after ligation of the femoral artery. This potent stimulator of TNF-a also markedly enhanced the number of monocyte-derived macrophages accumulated around growing collateral arteries. Peripheral and collateral conductance was markedly increased. Nevertheless, on a molar basis MCP-1 is the most potent arteriogenic peptide[21]. VEGF is a peptide with angiogenic properties. It is produced by cells in close vicinity of endothelial cells, its chemoattractive action on monocytes is dose dependent; and its expression is highly regulated by hypoxia and hence by a physiological feedback mechanism to tissue hypoxia[67].

2. Angiogenesis

2.1. The definition of angiogenesis

The term angiogenesis was introduced in 1935 by Hertig to describe the formation of new blood vessels in the placenta and, later, in 1971, by Folkman to describe the neovascularization accompanying the growth of solid tumors[21].

Angiogenesis, i.e., the sprouting of capillaries from the pre-existing vasculature, is mainly initiated by hypoxia in ischemic tissue[3]. These newly formed capillaries consist of endothelial tubes lacking proper wall structures. Angiogenesis alone has a limited capacity to increase perfusion of the surrounding ischemic tissue.

2.2. The process of angiogenesis

Angiogenesis is a process by which new capillary blood vessels sprout from a pre-existing blood vessel[68]. It is an important component of various normal and pathological conditions such as wound healing, fracture repair, folliculogenesis, ovulation, and pregnancy. These periods of angiogenesis are tightly regulated. However, if not properly controlled, angiogenesis can also represent a significant pathogenic component of tumor growth and metastasis, rheumatic arthritis, and retinopathies[21]. Angiogenesis is a complex phenomenon consisting of several distinct processes, which include endothelial migration and proliferation, extracellular proteolysis, endothelial differentiation (capillary tube formation), and vascular wall remodeling. It is important to recognize that these newly formed capillary tubes lack vascular smooth muscle cells.

2.3. The differences between arteriogenesis and angiogenesis

Arteriogenesis differs from angiogenesis in several aspects, **(Table 1.1., Fig.1.5.)** the most important being the dependence of angiogenesis on hypoxia and the dependence of arteriogenesis on inflammation[20]. However, angiogenesis and arteriogenesis share several mechanisms of action, e.g., their dependence on growth factors. Whereas angiogenesis can be largely explained by the actions of VEGF, arteriogenesis is probably a multifactorial process in which several growth factors are orchestrated. The role of VEGF in arteriogenesis is not clear, but a chemoattractive role for monocytes and hence an indirect contribution is imaginable[21].

Table1. 1.

Differences Between Angiogenesis and Arteriogenesis

	Angiogenesis	**Arteriogenesis**
Definition	Formation of new capillaries by sprouting	Growth of collateral arteries
Source	Pre-existing capillaries	Pre-existing arterioles
Location	Lower leg	Upper leg
Oxygen status	Hypoxia	Normoxia
Trigger	Ischemia	Shear stress
Cellular mechanism	Inflammation because of ischemic tissue damage	Inflammation because of focal increased shear stress
Increases blood flow maximally	1.5- to 1.7-fold	10- to 20-fold
Compensation for an occluded artery	Unable	Able

[20](Schaper, W. and Scholz, D., Arterioscler Thromb Vasc Biol, 2003; 23(7): 1143-51)

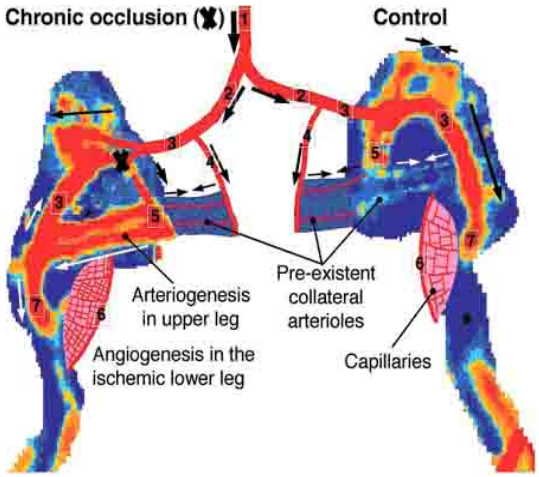
2.4. Conclusion

Arteriogenesis is by far the most efficient adaptive mechanism for the survival of ischemic limbs or internal organs such as heart and brain because of its ability to conduct, after adaptive growth, relatively large blood volumes per unit of time. An increase in the number of capillaries, the result of stimulated angiogenesis, is unable to

do that[21].

After peripheral artery occlusion in rabbits and mice, arteriogenesis proceeds much faster than angiogenesis because of a structural dilatation of pre-existing collateral vessels followed by mitosis of all vascular cell types, which restores resting blood flow within 3 days. Recovery of dilatory reserve (maximal flow) takes longer[20]. The slower angiogenesis is unable to significantly restore flow even if angiogenesis reduces the minimal terminal resistance of the entire chain of resistors by new capillaries in parallel. Future therapeutic efforts should be directed at stimulating arteriogenesis.

Fig. 1.5.



[20](Schaper, W. and Scholz, D., Arterioscler Thromb Vasc Biol, 2003; 23(7): 1143-51)

Fig.1.5. Collateral growth occurs in pre-existent arterioles. Laser Doppler image of collateral blood flow in an anesthetized mouse with exposed upper thigh skeletal muscles with chronic occlusion (7 days) of the left femoral artery. Shown are 2 pre-existent arterioles that exhibit a faintly visible flow signal but a very strong signal on the occluded side, i.e., the effect of growth after 7 days of occlusion. 1, indicates aorta; 2, iliac artery; 3, femoral artery; 4, A. pudenda externa; 5, A. profunda femoris; 6, A. tibialis posterior; and 7, A. saphena[20].

3. Gap junctions (Gj)

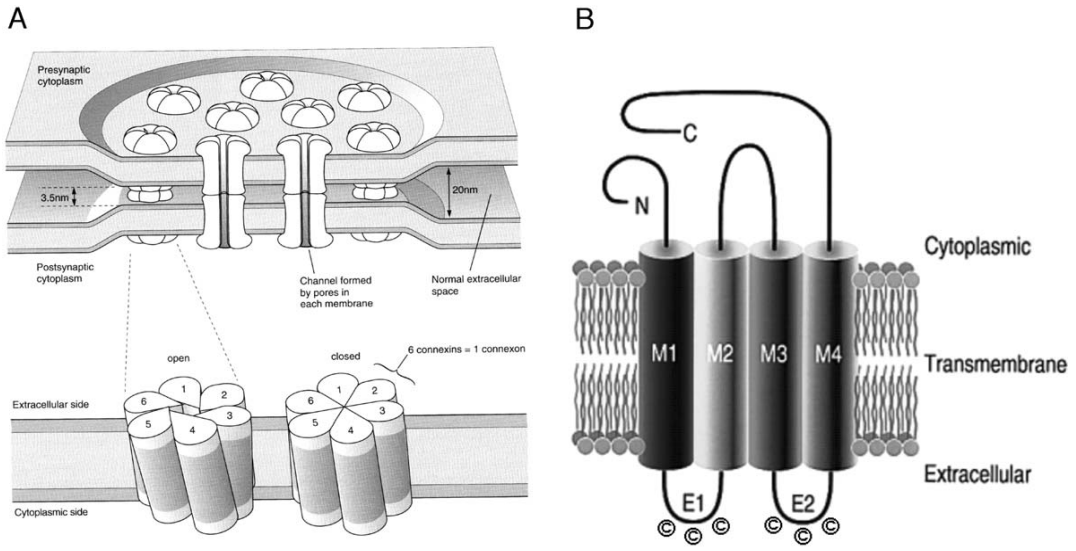
Intercellular communication is a key regulator of vascular function[69, 70]. In the vessel wall, cell-to-cell communication occurs by extracellular diffusion and convection of humoral factors or by intercytoplasmic exchange of ions, metabolites,

and small signaling molecules (<1 kDa) via gap junctions. Endothelial gap junctions are channels that permit and strictly regulate communication throughout the endothelial monolayer and between endothelial cells and adjacent smooth muscle and circulating blood cells. Endothelial cell migration and growth, particularly following injury and during angiogenesis, depend on communication through gap junctions[71-75]. In addition, gap junctions coordinate vascular tone and vasomotion [76-78] and participate in the regulation of immunoinflammatory responses[79, 80].

Connexins, or gap junction proteins, are a family of structurally-related transmembrane proteins that assemble to form vertebrate gap junctions (an entirely different family of proteins, the innexins, form gap junctions in invertebrates)[81]. Gap junctions are formed by a pair of hemichannels called connexons, each contributed by one of two neighboring cells. Connexons are composed of six connexin monomer subunits arranged around a central pore**(Fig.1.6.)**[82]. To date, the connexin gene family comprises 20 members in the mouse and 21 members in the human genome, 19 of which can be grouped as sequence-orthologous pairs. The structure of connexin genes is relatively simple. An untranslated exon 1 is separated by an intron of different length from exon 2, containing the uninterrupted coding region and the 3V-untranslated region (3V-UTR)[82].

Connexins are four-pass transmembrane proteins with both C and N cytoplasmic termini, a cytoplasmic loop (CL) and two extra-cellular loops, (EL-1) and (EL-2). Connexins are assembled in groups of six to form hemichannels, or connexons, and two hemichannels then combine to form a gap junction. They usually weigh between 26 and 60 kDa, and have an average length of 380 amino acids. The various connexins have been observed to combine into both homomeric and heteromeric gap junctions, each of which may exhibit different functional properties including pore conductance, size selectivity, charge selectivity, voltage gating, and chemical gating.

Fig.1. 6.

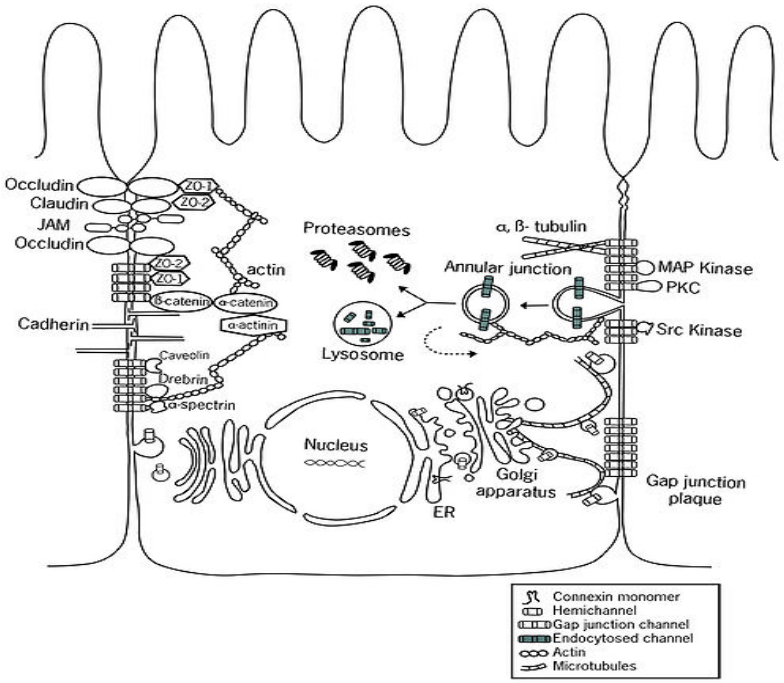


[82](Sohl, G. and K. Willecke, Cardiovasc Res, 2004; 62(2): 228-32)

Fig.1.6. (A) Schematic drawing of gap junction channels. Each apposed cell contributes a hemi-channel to the complete gap junction channel. Each hemichannel is formed by six protein subunits, called connexins. Six connexin subunits of the hemi-channel may coordinately change configuration to open and close the hemi-channel. Closure is achieved by connexin subunits sliding against each other and tilting at one end, thus rotating at the base in a clockwise direction. The darker shading indicates the portion of the connexon embedded in the membrane[82]. **(B)** Topological model of a connexin. The cylinders represent transmembrane domains (M1– M4). The loops between the first and the second, as well as the third and fourth transmembrane domains, are predicted to be extracellular (E1 and E2, respectively), each with three conserved cysteine residues[83].

Connexins 37, 40, and 43 (Cx37, Cx40, Cx43, respectively) are the major gap junction proteins expressed in vascular endothelial cells[85-89]. These proteins are very dynamic, exhibiting rapid turnover times and variable expression patterns. Because of the unique gating and permselective characteristics of Cx37, Cx40, and Cx43, different combinations of these connexin isoforms contribute to homo- or heteromeric connexons and homo- or heterotypic gap junctions leading to a variety of channel types with different functional properties[83, 90-96].

Fig.1.7.



[84](Dbouk HA, et al., Cell Commun Signal, 2009; 7: 4)

Fig. 1.7. Life cycle and protein associations of connexins. Connexins are synthesized on ER-bound ribosomes and inserted into the ER cotranslationally. This is followed by oligomerization between the ER and trans-Golgi network (depending on the connexin type) into connexons, which are then delivered to the membrane via the actin or microtubule networks. Connexons may also be delivered to the plasma membrane by direct transfer from the rough ER. Upon insertion into the membrane, connexons may remain as hemichannels or they dock with compatible connexons on adjacent cells to form gap junctions[84]. Newly delivered connexons are added to the periphery of pre-formed gap junctions, while the central "older" gap junction fragments are degraded by internalization of a double-membrane structure called an annular junction into one of the two cells, where subsequent lysosomal or proteasomal degradation occurs, or in some cases the connexons are recycled to the membrane (indicated by dashed arrow). During their life cycle, connexins associate with different proteins, including (1) cytoskeletal components as microtubules, actin, and actin-binding proteins α-spectrin and drebrin, (2) junctional molecules including adherens junction components such as cadherins, α-catenin, and β-catenin, as well as tight junction components such as ZO-1 and ZO-2, (3) enzymes such as kinases and phosphatases which regulate the assembly, function, and degradation, and (4) other proteins such as caveolin[84].

Although the extent of combinations of different connexins within connexons and

channels remains unclear, immunohistochemical and immunocytochemical studies demonstrate differential expression and localization patterns of all three vascular connexins in the endothelium, depending on species[97], vascular bed[98-100], and local hemodynamics[101]. In vivo studies implicate Cx40 as the constitutive vascular gap junction protein across species and vascular bed, playing an important role in coupling between cells in the vascular wall[97]. Gap junctions are essential for many physiological processes, such as the coordinated depolarization of cardiac muscle, and proper embryonic development. Cx37 is an early marker of arteriogenesis. It is normally not expressed in SMCs and only weakly in endothelium. Cx40 is strongly expressed in the working atrial myocytes, the central part of the atrioventricular (AV) node, the His bundle, and the distal elements of the cardiac conduction system (CS)[102]. It has never been detected in the ventricular myocytes. Cx40 is also expressed in the vasculature including many other tissues[102]. For this reason, mutations in connexin-encoding genes can lead to functional and developmental abnormalities. Null mutation of the Cx40 gene results in anomalies of impulse propagation at all levels of the CS, including the AV node, indicating that Cx40 plays a determinant functional role in electrical impulse transmission from the atria to the ventricles[103].

4. The femoral artery occlusion (FAO)

The occlusion of a major artery such as the femoral artery causes altered blood flow through pre-existing collateral arterioles. The endothelium is activated and the remodeling process of the vessel wall is initiated because of increased shear stress. The femoral artery occlusion model (FAO) is the standard model to investigate arteriogenesis/collateral growth. After the monitoring time of 1 to 21 days arteriogenesis has to be diagnostically confirmed via angiography including the measurement of vessels diameter (e.g. Microfil perfusion and MicroCT), blood flow measurement (e.g. laser Doppler Flow Imaging) and in vitro experiment (e.g. RT-PCR).

5. Laser Doppler Flow (LDF) Imaging

5.1. The definition of Laser Doppler Flow (LDF) Imaging

Laser Doppler is a standard technique for the non-invasive blood flow monitoring and measurement of blood flow in the microcirculatory system. The strength of the technique is in looking at changes in flow - either over time or differences in flow over an area of skin or other exposed tissue.

5.2. Operating principles

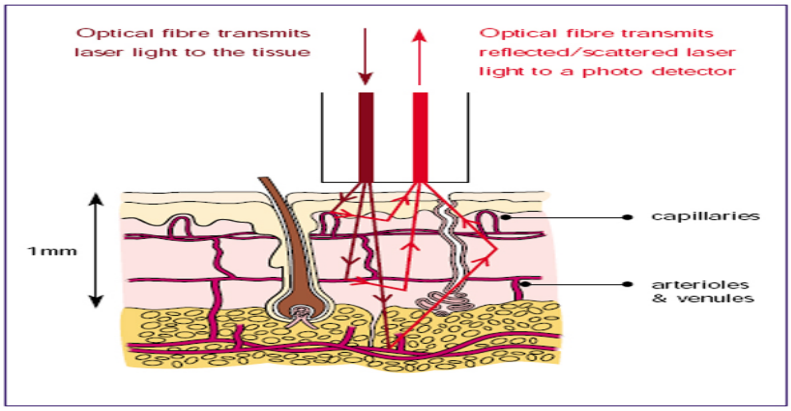
The laser Doppler technique measures blood flow in the very small blood vessels of the microvasculature, such as the low-speed flows associated with nutritional blood flow in capillaries close to the skin surface and flow in the underlying arterioles and venules involved in regulation of skin temperature. The tissue thickness sampled is typically 1mm, the capillary diameters 10 microns and the velocity spectrum measurement typically 0.01 to 10mm/s.

The technique depends on the Doppler principle whereby low power light from a monochromatic stable laser, e.g. a helium neon gas laser or a single mode laser diode, incident on tissue is scattered by moving red blood cells and as a consequence is frequency broadened. The frequency broadened light, together with laser light scattered from static tissue, is photo-detected and the resulting photocurrent processed to provide a blood flow measurement. Please note, where laser light is scattered for tissue with a low red blood cell concentration, the average Doppler frequency shift is proportional to the average speed of red blood cells.

Laser light can be directed to the tissue surface either via an optic fibre (**Fig.1.8.a**) or as a light beam (**Fig.1.8.b**). For ‘fibre optic’ monitors (**LDF** instruments) the optic fibre terminates in an optic probe which can be attached to the tissue surface. One or more light collecting fibres also terminate in the probe head and these fibres transmit a proportion of the scattered light to a photo-detector and the electronic signal processing

system. Normal fibre separations in the probe head are a few tenths of 1mm, consequently blood flow is measured in a tissue volume of typically 1mm^3 or smaller.

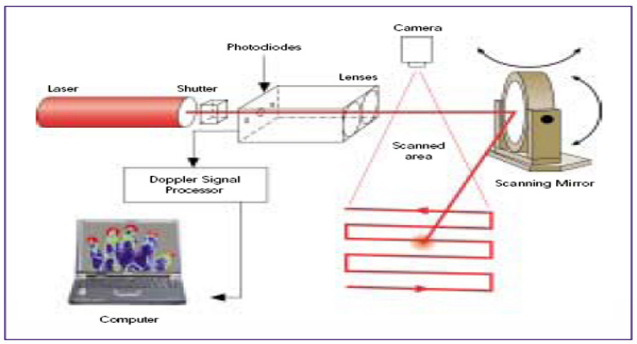
(**Fig.1.8.a**)



(Basic Theory and Operating Principles of LDF & LDI, Moor instruments Ltd User manual, 2003)

In a Laser Doppler blood flow Imager (LDI) the low intensity laser beam is scanned across a tissue surface in a raster fashion using a moving mirror. There is no direct contact with the tissue being assessed. The basic elements of the moorLDI are shown schematically in the following figure.

(**Fig.1.8.b**)



(Basic Theory and Operating Principles of LDF & LDI, Moor instruments Ltd User manual, 2003)

Both large areas (a full torso) and small areas (part of a finger) can be scanned, enabling the blood flow to be mapped and colour coded images of the blood flow displayed. Regions of interest can be defined and statistical data can be calculated and recorded.

Single point measurements give a high temporal resolution (40Hz data rates are typical) enabling rapid blood flow changes to be recorded, whereas the laser Doppler imager can provide spatial information and has the ability to average blood flow measurements over large areas. Fibre optic systems can measure at tissue sites not easily accessible to a laser beam. For example measurements in brain tissue, mouth, gut, colon, muscle and bone.

5.3. The definition of perfusion units

The term commonly used to describe blood flow measured by the laser Doppler technique is ‘flux’: a quantity proportional to the product of the average speed of the blood cells and their number concentration (often referred to as blood volume). This is expressed in arbitrary ‘perfusion units’ and is calculated using the first moment of the power spectral density.

6. Prospects and challenges

Cardiovascular diseases are the number one cause of death globally[104]. Arteries are the key vessels affected in cardiovascular diseases and the study of mechanisms of arterial growth and repair are, therefore, of fundamental interest. Arterial and venous vascular networks show a distinct genetic signature, function and branching architecture[105, 106]. Specification of arterial-venous vessel identity and formation of branched vascular networks occur during early embryogenesis and are modulated by hemodynamic factors[107-109], but the precise mechanisms are unclear. Circulation of blood creates mechanical forces in vessels[28, 110], and affects oxygenation of developing organs.

At present neuropilin-1[111, 112], ephrinB2[113-115], Unc5b[116], Notch1, Notch4, Jag1, Jag2 and Dll4[117-121], are established arterial markers. In the mouse, hemodynamic factors contribute to arterial-venous differentiation, involving regulation of arterial marker expression[108,109]. In the adult, hemodynamic factors regulate the enlargement and outgrowth of collateral arterial networks upon arterial stenosis[3, 11]. Several molecules originally described in embryonic arterial remodeling, also modulate the efficiency of arterial collateralization in the adult[12].

Besides these classical morphogenes, arteries also express the gap-junctional proteins Gja4 (Connexin-37) and Gja5 (Connexin-40)[69, 122-124]. Connexin 37 and Connexin 40 (Cx37, Cx40) are the major gap junction proteins expressed in vascular endothelial cells[85-89]. These proteins are very dynamic, exhibiting rapid turnover times and variable expression patterns. Although the extent of combinations of different connexins within connexons and channels remains unclear, immunohistochemical and immunocytochemical studies demonstrate differential expression and localization patterns of both vascular connexins in endothelium, depending on species[97], vascular bed[98-100], and local hemodynamics[101]. Gap junctional proteins mediate direct diffusion of signals between adjacent cells[41, 125]. For the microcirculation in vivo studies implicate Cx40 as the constitutive vascular gap junction protein across species

and vascular bed, playing an important role in coupling between cells in the vascular wall[78， 97-98], particularly in response to changes in tissue metabolic demand. The mechanism underlying arterial specific regulation of connexins is as yet unknown.

7. Objectives

Arteries are the key vessels affected in cardiovascular diseases and the study of mechanisms of arterial growth and repair are, therefore, of fundamental interest. Collateral artery growth is the most important tissue-saving, organ-saving, and often life-saving adaptive process after arterial occlusion in virtually all vascular provinces of the body. We hypothesized that arterial Gja5 expression plays a functional role in flow driven arteriogenesis. So we made use of the different kinds of mice for Connexin-40 mutant ($Gja5^{-/-}$), Connexin-40 floxed (Gja5f/f), and tamoxifen inducible Tie-2 Cre (Tie2CreERT2) and set up the mouse model for femoral artery occlusion. Then we assessed the blood flow with LDF and MicroCT imaging. We obtained genetic evidence in mice showing the functional importance of Gja5 in flow driven arterial remodeling and collateral arterial network development.

CHAPTER TWO: MATERIALS AND METHODS

1. Materials

1.1. Laboratory equipments

Balance(SG-2001)	Fisher Scientific, USA
Cryotome	LABEQUIP LTD., Canada
Dumont Angled Tip Forceps	Fine Science Tools, Switzerland
Dumont Curved Tip Serrated forceps	Fine Science Tools, Switzerland
Extra Fine Spring Scissors	Fine Science Tools, Switzerland
Freezer, -80 °C	Thermo Forma, Marlotta, USA
Fluorescent microscope	Leica Microsystems Switzerland Ltd
GFL Thermolab Shaking Water Bath - 1070	LabSource Limited, England
Hardened Fine Iris Scissors	Fine Science Tools, Switzerland
Heating pad	Mini Tub HT300
Intraoperative temperature control unit with temperature controlled heating plate	Moor Instruments, UK
Laser Doppler Flow imager	model LDI2-HR, Moor Instruments, UK
Leica KL1500LCD	Leica Microsystems Switzerland Ltd
Leica Microscope(M80)	Leica Microsystems Switzerland Ltd
MicroCT	Inveon, Siemens, Germany

Microfil	MV-122; Flow Tech Inc., Carver, MC，USA
Operating table	Moor Instruments, UK
Personal computer	Dell, USA
Pipettes, adjustable	Eppendorf AG, Hamburg, Germany
Needle holder	Fine Science Tools, Switzerland
Refrigerator	Liebherr comfort, Germany
Scantainer	Scanbur Ltd, Denmark
Sequence Detection System 2.3	Applied Biosystems, Foster City, USA
Stereotactic microscope	Leica Microsystems Switzerland Ltd
VV3 S40	VWR laboratory equipment, USA
7900HT Fast Real-time PCR System	Applied Biosystems, Foster City, USA

1.2. Chemicals, reagents, primers and probes, kits and other materials

1.2.1.Chemical and reagents:

Bepanthene ophthalmic ointment	Bayer Vital GmbH, Germany
Ethanol, 100%	Sigma, Deisenhofen, Germany
Heparin solution (1,000 USP unit/ ml)	Baxter Health Care, Braun, Germany
Hair removing crème	Dirk Rossmann GmbH, Germany
100 mg/ml ketamine	Pfizer Pharmacia GmbH, USA
Liquid nitrogen	Linde，Germany
Papaverine hydrochloride (100	Paveron, Weimer, Germany

mg/kg)	
Paraformaldehyde	Sigma, Steinheim, Germany
Phosphate buffered saline (PBS)	Biochrom, Berlin, Germany
Injection grade sterile saline solution, 0.9% (wt/vol) sodium chloride	Fresenius Kabi Deutschland GmbH, Germany
Tamoxifen	Sigma, Deisenhofen, Germany
Trizol	Invitrogen, Heidelberg, Germany
Xylazine (2% Rompun)	Bayer Vital GmbH, Germany

1.2.2. Primers and probes (BioTez GmbH, Berlin, Germany)

Gja5 Murine real-time PCR assay (5′to 3′)	Forward primer: CAGCCTGGCTGAACTCTACCA Reverse primer: CTGCCGTGACTTGCCAAAG TaqMan probe: CGCTGTCGGATCTTCTTCCAGCCCAG
Gja5 ko genotyping primers (5′to 3′)	Forward primer: tggagccacagttgcaatggt Reverse primer (wt): tctctgactccgaaaggcaag Reverse primer (ko):gcacgagactagtgagacgtg
Gja5 conditional ko genotyping primers (5′to 3′)	Forward primer: GGCCATCCTCTGCTACATATGCAG Reverse primer : GTGACATGACCTGGATCTCTGGAG

1.2.3. Kits and other materials

Aluminiumfolie	Carl Roth GmbH, Germany
BD Falcon™ Polypropylene Conical Tubes (15ml)	BD Sciences, Franklin Lakes, USA

BD Falcon™ Polypropylene Conical Tubes (50ml)	BD Sciences, Franklin Lakes, USA
Cotton-tipped applicators	Wilh weisweiler GmbH, Germany
Double-sided sticky tape from office supply stores	Germany
Destruction bags	VWR international GmbH, Germany
Examination Gloves	Sanger GmbH, Germany
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, Foster City, USA
qPCR MasterMix Plus for SYBR® Green I dNTP	Eurogentec, San Diego, USA
Syringes (1 ml)	Braun, Germany
Syringes (2 ml)	Braun, Germany
Syringes (20 ml)	Braun, Germany
Sterile artery ligation thread (Polyester S-black, 3/0 USP)	SMI, Belgium
Sterile artery ligation thread (Polyester S-black, 5/0 USP)	SMI, Belgium
Sterile surgical suture material (Polyester S-black, 7/0 USP)	Johnson & Johnson Healthcare Company, USA
Surgical knife	Fine Science Tools, Switzerland
20-G needle	BD Biosciences, USA
21-G needle	BD Biosciences, USA
25-G needle	BD Biosciences, USA

1.3. Experimental Animals

To study the function of Gja 5 in mouse arteriogenesis, I used Gja 5 knockout mice

(both conventional and conditional) and Gja 5 EGFP (CX40EGFP) reporter mice in the experiments.

Connexin-40 mutant ($Gja5^{-/-}$), Connexin-40 floxed (Gja5f/f), and tamoxifen inducible Tie-2 Cre (Tie2CreERT2) mice have already been described[126-128]. To generate tamoxifen-inducible endothelial cell specific Gja5 mutant mice, we mated the Tie2CreERT2 transgenic mice with Gja5f/f mice. For induction of Cre activity, mice homozygous for floxed Gja5 (Gja5f/f) and carrying the Tie2CreERT2 transgene (Tc^{+}), as well as their Cre-negative (Tc^{-}) littermates, were injected with tamoxifen i.p. (30 mg/kg b.w.), once a day, for a period of two weeks, before FAO.

The enhanced-green fluorescent protein (EGFP) gene was inserted into the 2^{nd} exon of CX40 gene, so the EGFP expression is under the control of the Cx40 gene in the Connexin-40 EGFP reporter mice($Cx40^{EGFP/+}$ mice), as described[126].

The conventional ko mice were divided into three groups: $Gja5^{-/-}$ group (Cx40 ko), $Gja5^{+/-}$ group (Cx40 Het) and $Gja5^{+/+}$ group (wt); while the conditional ko mice were divided into Tie2 Cre^{+} ERT2 Gja5f/f (ko) and Tie2 Cre^{-} ERT2 Gja5f/f (control) groups.

All the procedures followed in the care and euthanasia of mice was in accordance with the European Community Standards on the Care and Use of Laboratory Animals, and the protocol was approved by the local ethics committee.

1.4. Software

Amira software 5.2.2.	Visage Imaging GmbH, Germany
Primer Express™ Version 2.0.0	Applied Biosystems, Foster City, USA

2. Methods

2.1. Femoral artery occlusion (FAO) model

Femoral artery occlusion results in flow driven formation of an arterial collateral network which increases blood flow to the ischemic hindlimb[129]. Occlusion of the right femoral artery in 12 weeks old mice was performed as described[130]. **(Fig.2.1.)**

2.1.1. Anesthesia

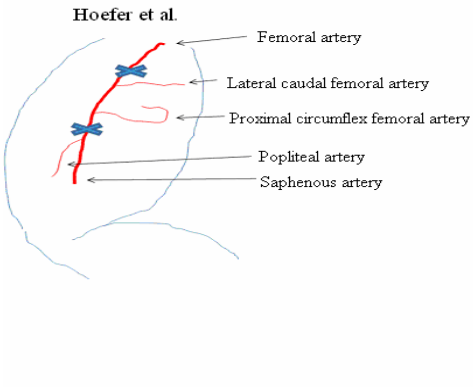
The mouse was anesthetized with an intraperitoneal injection of 100 mg/kg ketamine (100mg/ml) and 10 mg/kg xylazine (20mg/ml) and placed in supine position. Both feet were fixed with adhesive tape on a 37°C temperature heating pad. Bepanthene ophthalmic ointment was applied to both eyes of mouse for protection.

2.1.2. Surgery

The right inguinal area was cleaned from hairs with hair removing cream and disinfected with 70% ethanol. After disinfection, the femoral artery was seen. Parallel to this vessel, a 0.5 cm incision was made on the thigh skin. The anatomical landmarks were identified with neurovascular bundle of femoral nerve, artery and vein. The connective tissues among the bundle of femoral artery, vein and nerve were gently tugged and slightly stretched with Dumont Angled Tip Forceps. The femoral artery was exposed and separated from vein and nerve.

Both two ligations had to be done for the FAO according to Hoefer's method[130]. The proximal circumflex femoral artery is very closely connected to the lateral caudal femoral artery. The upper ligation is done proximally to both branches. The second ligation is done below both branches. But it should be noted that the femoral artery is then split into the saphenous and popliteal artery. The second ligation is placed proximally of this position. The femoral artery is occluded using triple surgical knots followed by closing of the wounds.

Fig. 2.1.



[130](Hoefer, I. E., et al., Circ Res, 2004; 94(9): 1179-85)

2.2. Assessment of blood flow with Laser Doppler Flow (LDF) Imaging

For repeated assessment of hindlimb blood flow after FAO, we used the non-invasive LDF imaging technique[129]. The Doppler signal is linearly proportional to perfusion of the upper 200–300 μm of the skin[131]. Tissue perfusion is quantified in regions of interest (ROI) defined in the limbs relative to the contralateral, non-ligated side and can be displayed as color-coded images[132]. Perfusion measurements obtained from ROIs of thighs are confounded by fur, skin pigmentation and motion artifacts from the abdomen and have been shown not to correlate with limb perfusion[132]. We therefore take LDI measurements from the feet, which correlate with other measures of limb perfusion[18, 133]. After animals were anesthetized, perfusions of both hindlimbs were obtained separately before FAO, acutely after FAO, at 1, 3, 7, 14, 21 days after FAO using a scanning Laser Doppler Flow Imager.

2.2.1. Starting the moor LDI2 system

·Switch on the moor LDI2 system at the control box; turn on the PC and monitor;

·Observe laser warm up time: 30 minutes;

·Load the moorLDI2 software and then click on Measurement

2.2.2. Mouse preparation

·Measure the weight of mouse; write down its number and weight; intraperitoneal anesthesia according to its weight;

·Lay the mouse on a special operating table in supine position, stretch its feet and tape them to the surface of the table; put the small table under the laser beam.

2.2.3. Basic configuration

·Configure the scan; configure distance and video settings;

·Repeat image mode configuration.

2.2.4. Scan

·Click Repeat Scan Window; Distance Check; Start scan; Mostly, I scan five images per one measurement.

·After scan is complete, click on “File” and the “Save as”.

2.2.5. Analyze

·Load the moorLDI2 software and then click on Image Review.

·Open the file for LDF Image; Measure the Region for LDF Image;

·Analyze the flux with the moorLDI2 software.

2.3. Micro-Computed Tomography (MicroCT) Imaging

For visualization of the arterial collateral network after FAO, we used MicroCT imaging. At 7 days after FAO, the abdominal aorta was cannulated, and perfused with 100 mg/kg papaverine hydrochloride to obtain maximal vasodilation, followed by perfusion with Microfil. Collateral arterial network morphology was analyzed with Amira software 5.2.2 after PET/ CT scanning with a resolution of 20μm[134, 135]. All measurements were taken directly from the 3D reconstruction. Detection of collateral arteries: only arterial connections with a stem (input connection), midzone and re-entry (output connection) where analyzed as true collateral arteries. Three consecutive measurements were taken in the midzone of these collateral arteries. Collaterals typically showed a tortuous appearance.

2.4. In vitro experiment

The mice were killed with cervical dissociation. The skin and fasciae were removed from the thighs and lower limbs of the ligated and non-ligated sides of the animal.

The gastrocnemius (GC) muscle was isolated and excised, then it was immediately frozen in liquid nitrogen and stored at -80°C Freezer. Total RNA of the GC muscle was isolated using TRIzol reagent. Real-time RT-PCR was performed using the TaqMan probe-based chemistry. Primers and probes were designed using the Primer Express 2.0 software; sequences are shown in **Fig.3.4.** (**B**). Real-time PCR amplification reaction was performed on a Sequence Detection System using the Taqman gene expression MasterMix, according to manufacturer's instructions. Reactions were performed in triplicate. Data were collected and analyzed with the Sequence Detection System 2.3 software. The relative amount of mRNA was calculated after normalization to glyceraldehyde phosphated dehydrogenase (GAPDH).

2.5. Statistical analysis

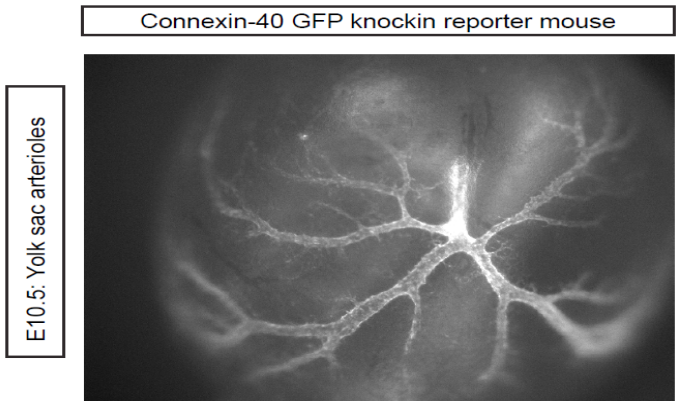
Data are presented as mean ± SEM. P values were calculated using paired Student's t test and the Mann Whitney U test (for non-normal distributions). Differences were considered to be significant at $p < 0.05$.

CHAPTER THREE: RESULTS

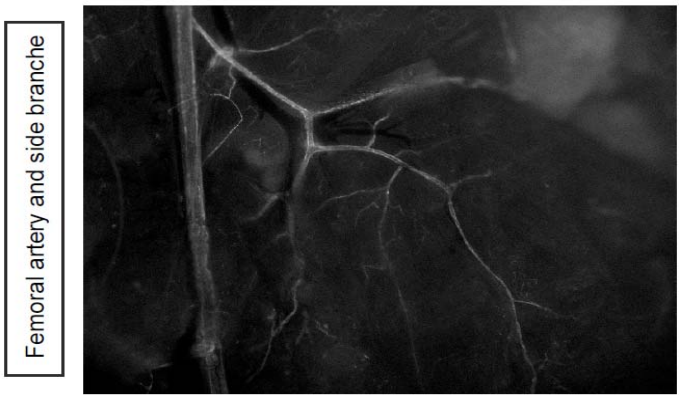
1. The Gja5 (connexin-40) was expressed in mouse arteries

Using CX40EGFP reporter animals, I found that the CX40 was expressed in the arteries, both in the embryos and in the adults. **Fig.3.1** Imaging arterial expression of Gja5 (connexin-40) (**A**) yolk-sac (E10.5), (**B**) hindlimb and (**C**) brain anterior cerebral artery (ACA); middle cerebral artery (MCA); posterior communicating artery (PComA).

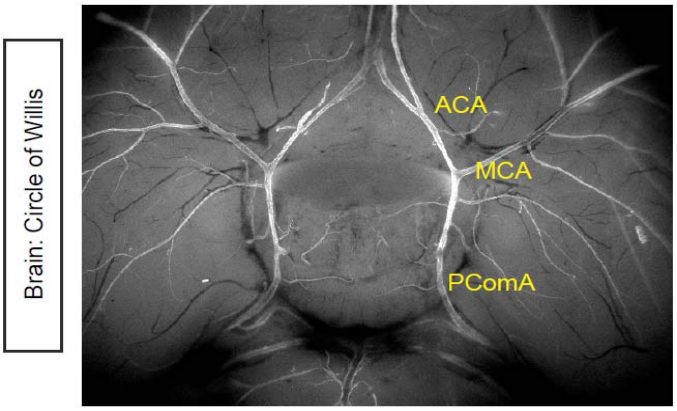
Fig.3.1.(**A**)



(**B**)



(**C**)



2. More reduction of hindlimb perfusion has been found in Gja5 deficient mice after FAO

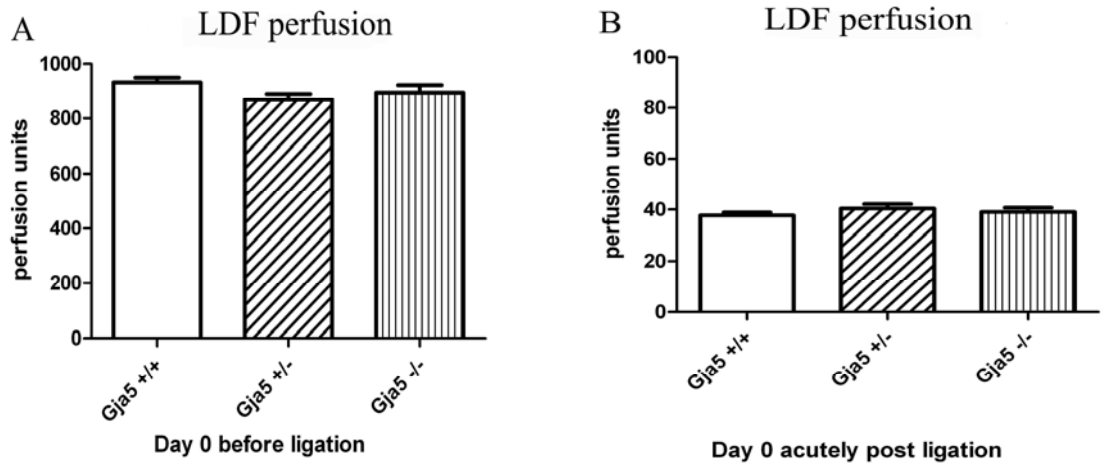
To test whether Gja5 plays a functional role in flow mediated adaptive remodeling of arteries, I used the flow driven arteriogenesis model (the hindlimb femoral artery occlusion model) and investigated Gja5 mutant mice, both conventional and conditional.

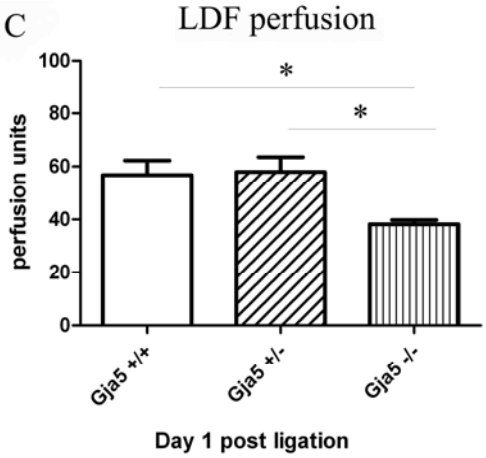
To check the hindlimb perfusion after FAO, I measured the hindlimb perfusion with LDF imaging at the following time-points: before FAO, acutely after FAO, day 1, day 3, day 7, day 14 and day 21. The LDF imaging showed that there was more reduction of hindlimb perfusion in Gja5 deficient mice compared to the $Gja5^{+/+}$ animals after FAO (**Fig. 3.2.c-d**). Gja5 expressed in arterial endothelium mediates vasodilatory responses. To examine the involvement of this functional component, we examined mice in which we deleted Gja5 expression specifically from the endothelium before FAO (**Fig. 3.2.f-j**). For this purpose, Gja5f/f mice were mated with endothelial specific tamoxifen inducible Tie-2 Cre mice. But in the conditional ko mice, only a small reduction of hindlimb perfusion was found in the ko group on day 3. For details please see Fig 3.2.

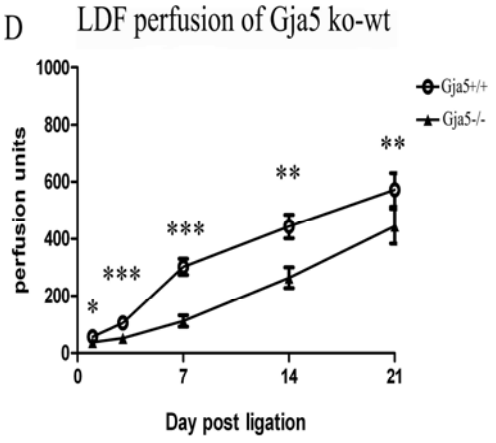
Fig.3.2. (**A-C**) Hindlimb perfusion in $Gja5^{+/+}$, $Gja5^{+/-}$ and $Gja5^{-/-}$ mice; (A) there was no significant difference for hindlimb perfusion among $Gja5^{+/+}$, $Gja5^{+/-}$ and $Gja5^{-/-}$ mice before FAO, (B) there was no significant difference for hindlimb perfusion among $Gja5^{+/+}$, $Gja5^{+/-}$ and $Gja5^{-/-}$ mice acutely after FAO, (C) there was significant reduction for hindlimb perfusion in $Gja5^{-/-}$ mice compared to $Gja5^{+/+}$ mice at 1 day after FAO. (**D**) There were significant reductions for hindlimb perfusion in $Gja5^{-/-}$ mice compared

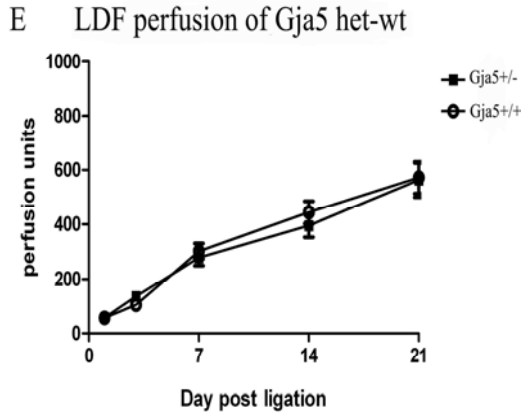
to $Gja5^{+/+}$ mice separately at 3 days, 7 days，14 days and 21 days after FAO. (**E**) There was no difference between $Gja5^{+/-}$ mice and $Gja5^{+/+}$ mice. (**F-J**) Effects of inducible endothelial specific deletion of Gja5 on hindlimb perfusion; Tc^{+} Gja5f/f: inducible Cre^{+}; Gja5f/f mice treated with tamoxifen. Tc^{-} Gja5f/f: inducible Cre^{-}; Gja5f/f mice treated with tamoxifen. (F) there was no significant difference for hindlimb perfusion between Tc^{+} Gja5f/f mice and Tc^{-} Gja5f/f mice before FAO, (G) there was no significant difference for hindlimb perfusion between Tc^{+} Gja5f/f mice and Tc^{-} Gja5f/f mice acutely after FAO, (H) there was no significant difference for hindlimb perfusion between Tc^{+} Gja5f/f mice and Tc^{-} Gja5f/f mice at 1 day after FAO. (I) at 3 days after FAO, a small reduction was noted, but perfusion was restored to normal afterwards. (J) there was no significant difference for hindlimb perfusion between Tc^{+} Gja5f/f mice and Tc^{-} Gja5f/f mice, at 7 days, 14 days and 21 days after FAO. (**K**) LDF imaging at 3 days after FAO showed that hindlimb perfusion remained reduced in $Gja5^{-/-}$ mice compared to $Gja5^{+/+}$ mice ($p<0.001$), but there was no difference between $Gja5^{+/-}$ mice and $Gja5^{+/+}$ mice when compared to $Gja5^{+/+}$ mice or $Gja5^{+/-}$ mice; (**L**) LDF imaging at 7 days after FAO showed that hindlimb perfusion remained reduced in $Gja5^{-/-}$ mice compared to $Gja5^{+/+}$ mice ($p<0.001$), but there was no difference between $Gja5^{+/-}$ mice and $Gja5^{+/+}$ mice.

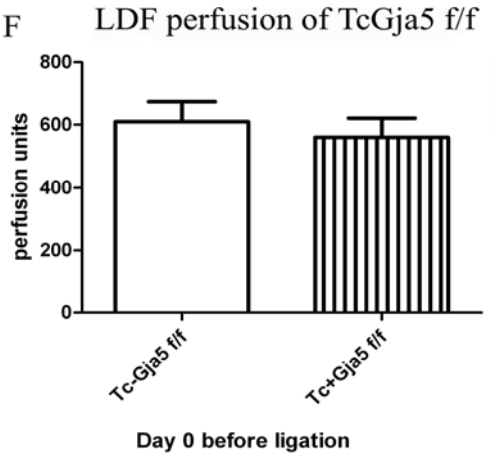
*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

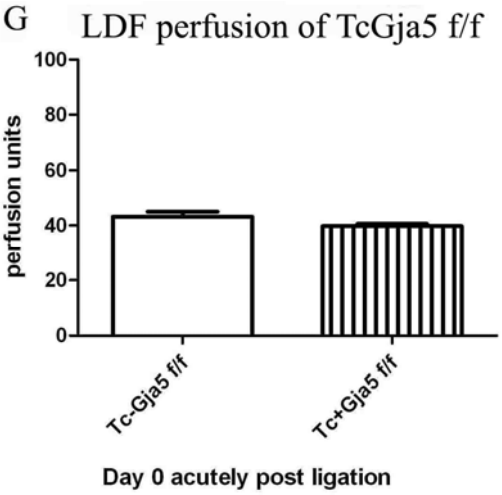


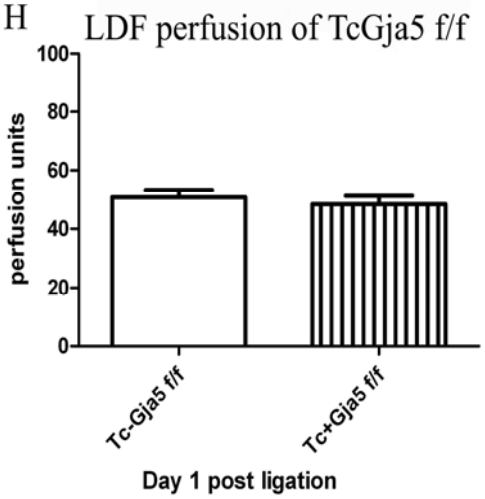

C
LDF perfusion
perfusion units
100
80
60
40
20
0
*
*
Gja5 +/+
Gja5 +/-
Gja5 -/-
Day 1 post ligation

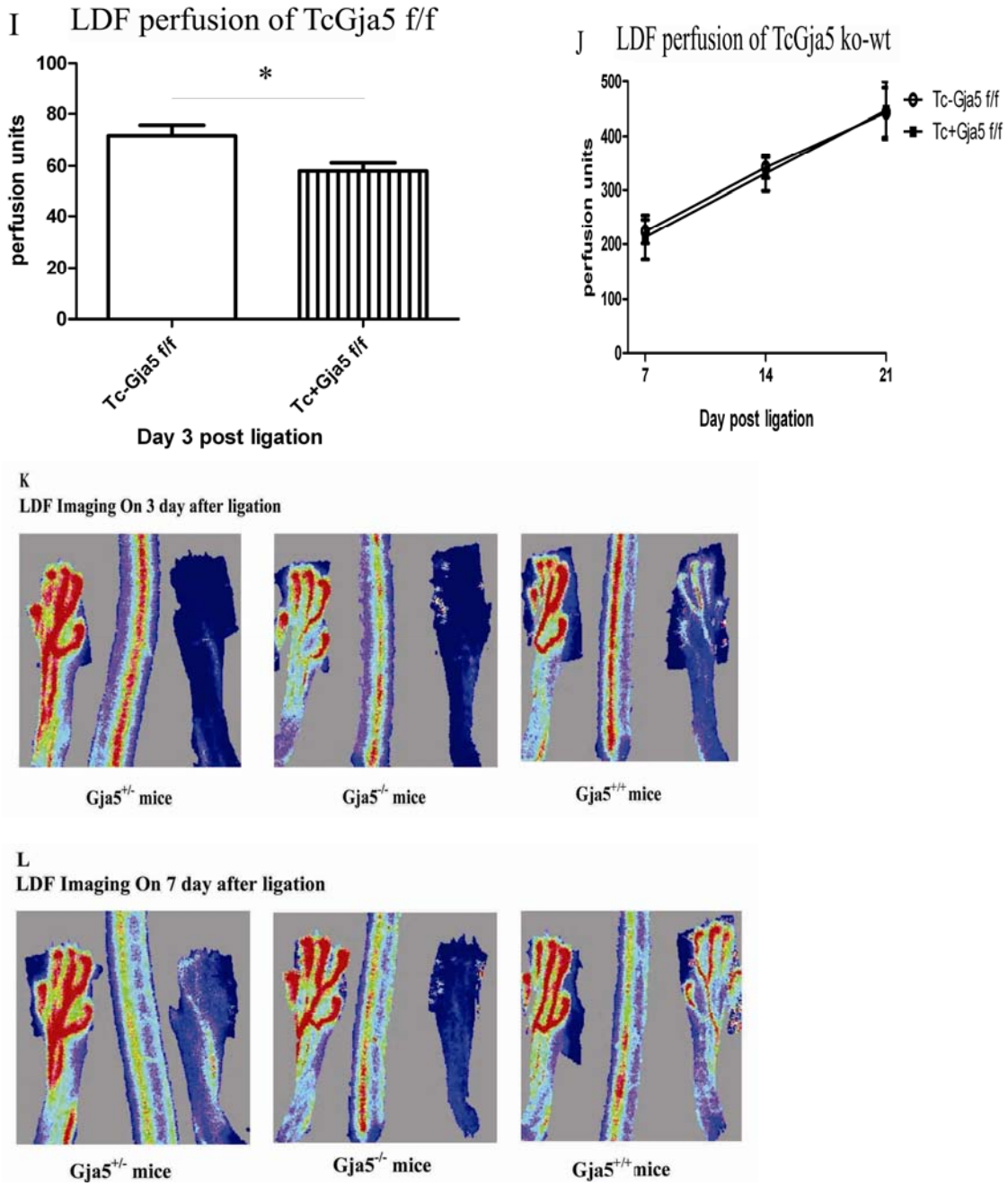

D
LDF perfusion of Gja5 ko-wt
perfusion units
1000
800
600
400
200
0
Gja5+/+
Gja5-/-
*
***
***
**
**
0
7
14
21
Day post ligation


E
LDF perfusion of Gja5 het-wt
perfusion units
1000
800
600
400
200
0
Gja5+/-
Gja5+/+
0
7
14
21
Day post ligation


F
LDF perfusion of TcGja5 f/f
perfusion units
800
600
400
200
0
Tc-Gja5 f/f
Tc+Gja5 f/f
Day 0 before ligation


G
LDF perfusion of TcGja5 f/f
perfusion units
100
80
60
40
20
0
Tc-Gja5 f/f
Tc+Gja5 f/f
Day 0 acutely post ligation


H
LDF perfusion of TcGja5 f/f
perfusion units
100
80
60
40
20
0
Tc-Gja5 f/f
Tc+Gja5 f/f
Day 1 post ligation



3. Smaller collateral arterial networks have been found in Gja5 deficient mice after FAO.

In mice, the blood flow is shunted through pre-existing collateral arteries that bypasses the occlusion after FAO (**Fig. 3.3.a**). Increased blood flow causes outward remodeling which results in diameter increase and leads to restoration of perfusion to the ischemic regions. The efficacy to transport blood into the ischemic hindlimb depends on the size and number of the collateral arteries. To address this, we perfused the hindlimbs of

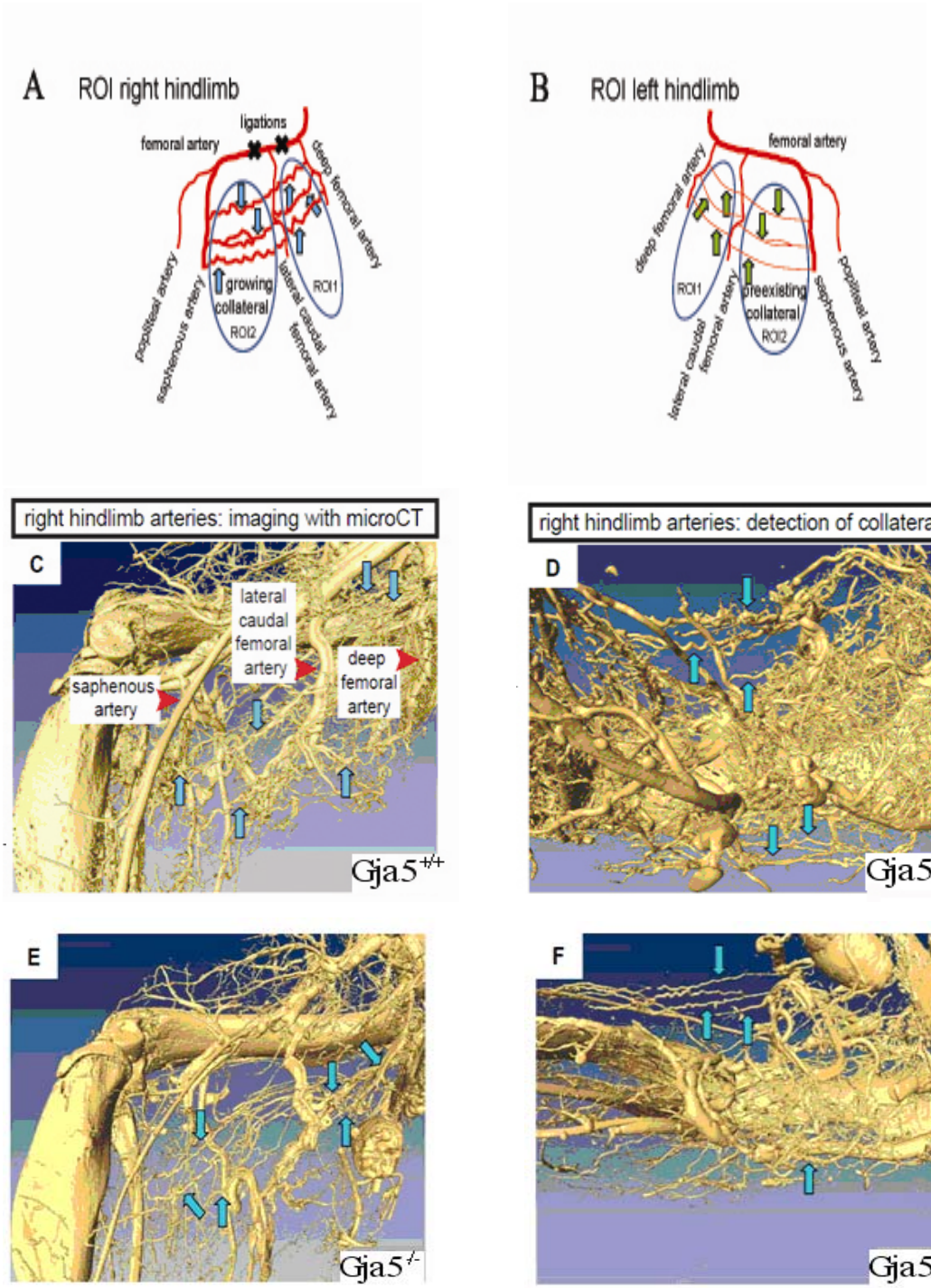
Gja5$^{-/-}$ mutant and Gja5$^{+/+}$ mice with contrast agent (Microfil) under maximal vasodilatory conditions, and imaged the morphology of the collateral arterial network at 7 days after FAO using MicroCT (**Fig. 3.3.a-f**). Seven days after FAO, smaller collateral arterial networks were found in Gja5 deficient mice compared to the Gja5$^{+/+}$ animals in MicroCT imaging ($P<0.05$) (**Fig. 3.3.g**), and there was no difference in the diameters of pre-existing collaterals between Gja5$^{-/-}$ and Gja5$^{+/+}$(**Fig. 3.3.h**).

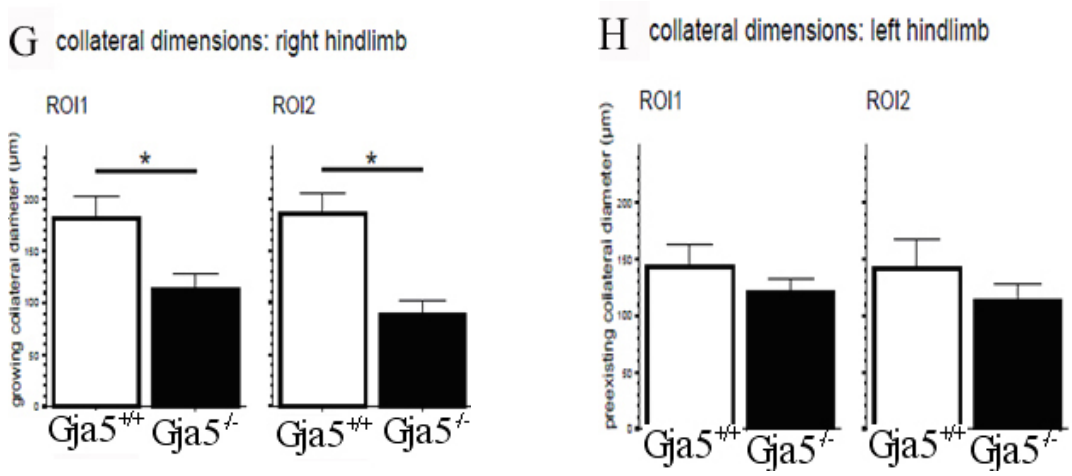
Arterial collateral diameters were significantly smaller ($p<0.05$) in Gja5$^{-/-}$ mice (n=5) when compared to Gja5$^{+/+}$ mice (n=4) (**Fig. 3.3.g, h**). The total number of growing collaterals was smaller in Gja5$^{-/-}$ (3.8±0.37) mice when compared to Gja5$^{+/+}$ (6.0±0.81) mice. To image vessels below the detection limit of the MicroCT, we used histological analysis of the gastrocnemius muscle. We observed significantly smaller lumen diameters in Gja5$^{-/-}$ mice when compared to Gja5$^{+/+}$ mice (17.23±0.51µm in Gja5$^{-/-}$ versus 23.23±1.01µm in Gja5$^{+/+}$, $p<0.05$; Gja5$^{-/-}$ 4 animals/group, 4 sections/muscle; Gja5$^{+/+}$ 3 animals/group, 4 sections/muscle; in total about 250 vessels for Gja5$^{-/-}$ or Gja5$^{+/+}$ were measured). Taken together: Gja5$^{-/-}$ mutants showed smaller and fewer collateral arteries after FAO.

Fig.3.3. (**A, B**) Schematic overview of the regions of interest in the occluded right hindlimb (A) and control left hindlimb (B). (A) The blood flow is shunted through pre-existing collateral arteries after FAO. Increased blood flow causes outward remodeling which leads to a diameter increase. The growing arterial collaterals (A, blue arrows) in region of interest -1 (ROI-1) were detected between the deep femoral artery and the lateral caudal femoral artery, and there is region of interest -2 (ROI-2) between the lateral caudal femoral artery and saphenous artery. Pre-existing collateral arteries in the control hindlimb (B, green arrows) were analyzed at anatomically comparable positions. Representative MicroCT scans showed the arterial network at 7 days after FAO in the right hindlimb of Gja5$^{+/+}$ (**C, D**) and Gja5$^{-/-}$ mice (**E, F**). (C, E) Overview images showed the position of the major arteries (red arrowheads with legends), and arterial collaterals (blue arrows) in the occluded right hindlimb of Gja5$^{+/+}$ and Gja5$^{-/-}$ mice. (D, F) The growing collaterals (blue arrows) can be detected in the occluded right

hindlimb of Gja5$^{+/+}$ mice and Gja5$^{-/-}$ mice. (**G**) Collateral arterial diameters were significantly smaller in Gja5$^{-/-}$ mice compared to Gja5$^{+/+}$ mice. (**H**) There was no difference in diameters of pre-existing collateral arteries between Gja5$^{-/-}$ mice and Gja5$^{+/+}$ mice.

* $p<0.05$, t-test.



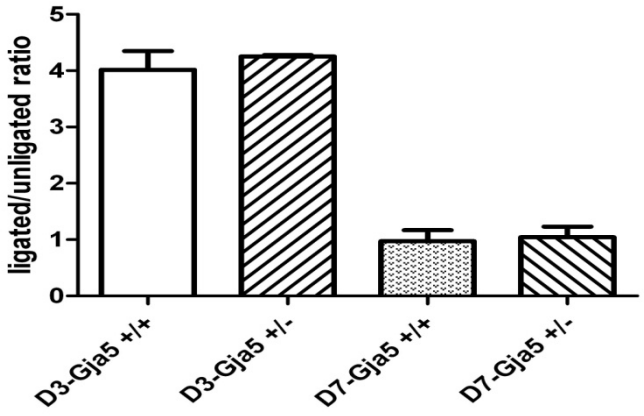


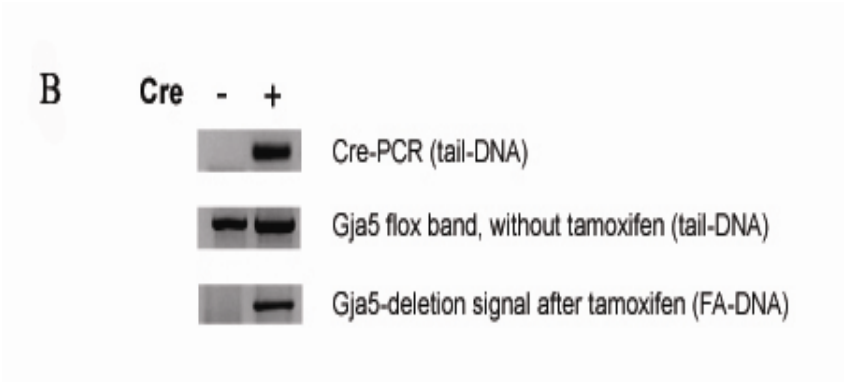
4. Expression of Gja5 in the femoral artery and gastrocnemius muscle after FAO

Using real-time PCR, I measured the expression of the Gja5 in the experimental animals.

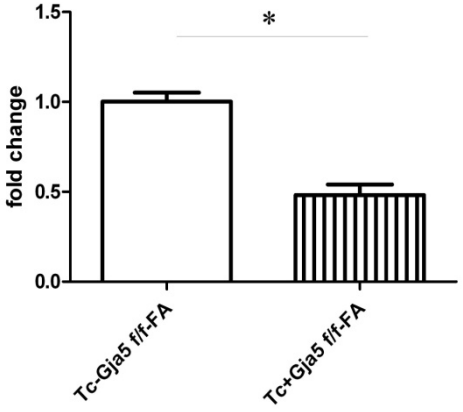
Fig.3.4. (**A**) Changes of Gja5 expression in gastrocnemius muscle after FAO. The expression of Gja5 is shown as ratio from expression in the ischemic hindlimb and the control hindlimb, using the gastrocnemius muscle of $Gja5^{+/+}$ and $Gja5^{+/-}$ mice (n=3 animals per group). The expression levels of Gja5 in $Gja5^{+/-}$ mice and $Gja5^{+/+}$ mice were both 4-fold higher in the ischemic hindlimb at 3 days after FAO. Then the expression levels of Gja5 returned to baseline values at 7 days after FAO. (**B, C, D**) Expression of Gja5 was reduced after endothelial specific deletion of Gja5. Tie2CreERT2 x Gja5f/f mice were treated with tamoxifen once daily for 2 weeks before FAO. Then expression of Gja5 was measured in the femoral artery and gastrocnemius muscle. (B) the Gja5-deletion signal could be detected by genotyping PCR in the femoral artery DNA after tamoxifen treatment. (C) the real-time PCR showed that there was a significant difference in the expression of Gja5 in the femoral artery between Tc^{+} Gja5f/f mice and Tc^{-} Gja5f/f mice after tamoxifen treatment. (D) there was a significant difference in the expression of Gja5 in the right gastrocnemius muscle (ligated leg) between Tc^{+} Gja5f/f mice and Tc^{-} Gja5f/f mice after FAO. * $P<0.05$, n=3 animals per group, t-test.

A Expression of Gja5 in R-Gestrocnemius muscle after ligation

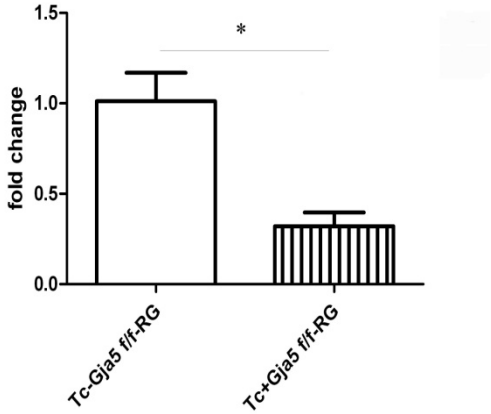




C Expression of Gja5 in FA



D Expression of Gja5 in R-Gastrocnemius muscle



CHAPTER FOUR: DISCUSSION

Arteriogenesis (collateral vessel growth) is triggered by fluid shear stress in case of an arterial occlusion. It is an important focus in current cardiovascular research as it might provide new therapeutic opportunities. But the molecular mechanisms underlying arteriogenesis are not yet completely understood. Gap junctions play a multifaceted role in the vasculature that is essential in the control of gene expression, vascular development, and vascular function. However, the function of gap junctions in the vasculature does not depend only on the molecular selectivity or permeability of the different vascular connexin isoforms. It is well established that neurogenesis and angiogenesis share common molecules and mediators[136], including the principle of electrical coupling involving gap junction proteins or connexins[125]. Although the participation of the gap junction in vascular function seems to be very complex, the development of connexin-knockout animals has been a great contribution to our understanding of how these proteins work in the vasculature.

In addition, connexin-mimetic peptides have been demonstrated to be an effective tool to dissect the participation of gap junctions in vascular function. Connexins permit crosstalk between adjacent cells via the rapid exchange of ions and small metabolites. Arteries most notably express connexin-37 (Gja4) and connexin-40 (Gja5)[125]. Gja5 and Gja4 mutant mice are viable, but the double knockout mice die in utero and show the other angiogenic remodeling defects[124]. Gja5 is preferentially expressed in endothelium, and mice deficient in Gja5 show impaired conduction of endothelium dependent vasodilator responses along arterioles[78].

The present study demonstrates that Gja5 may function as a positive modulator of arteriogenesis and serve as an arterial marker. We could show that: 1) Gja5 plays a functional role in arteriogenesis; 2) smaller and fewer collateral arteries in Gja5$^{-/-}$ mice; 3) Gja5 conditional mutant mice had a small perfusion reduction at 3 days after FAO; 4) reduced Gja5 in endothelial cells may play a functional role in arteriogenesis.

1. Gja5 plays a functional role in arteriogenesis.

To prove the potential role of Gja5 in arteriogenesis, we investigated Gja5 mutant and Gja5 conditional mutant mice using established flow driven arteriogenesis models, the femoral artery occlusion model[129]. Surgical ligation of the femoral artery at a specific site triggers arteriogenesis of small, pre-existing collateral arteries into functional conduit vessels proximally and ischemic angiogenesis distally. The vascular response to hind-limb ischemia can be readily evaluated by laser Doppler-based perfusion measurements, histological quantification of arteriogenesis and MicroCT imaging.

Our model is similar to vascular development to some extent because of the similarity of the hemodynamic situation. The occlusion of a major supply artery can result in the formation of an endogenous arterial bypass circulation that restores perfusion to the ischemic region[20]. The efficacy of this process depends on the number of pre-existing collateral arterioles and their capacity to remodel outward into larger caliber arteries, as initiated by increased blood flow and shear stress[11]. Because of the existence of pre-existing small collateral vessels, a significant degree of shear stress might be much easier and more rapidly established in the vessels of the region governed by the chronically occluded artery. It has been shown that after occlusion of the femoral artery, blood flow is shunted to pre-existing collateral arterial pathways around the occlusion. Increased fluid shear stress stimulates structural diameter increase of these small arteries[11]. As a result of this remodeling process, blood flow to the ischemic region is progressively restored. This process is also known as collateral arteriogenesis[20].

Gja5 was expressed in the femoral artery. We measured blood flow by Laser Doppler Flow imager and showed a significantly reduced perfusion between day 1 and day 21 after FAO in $Gja5^{-/-}$ mice compared to $Gja5^{+/+}$ mice. But there was no significant difference in perfusion reduction after FAO between $Gja5^{+/-}$ mice and $Gja5^{+/+}$ mice. This indicated that Gja5 plays a functional role in arteriogenesis.

2. Smaller and fewer collateral arteries in $Gja5^{-/-}$ mice.

Studying vascular development or the mechanisms of neovascularisation (angiogenesis, arteriogenesis or vasculogenesis) and evaluating the effects of pro or antiangiogenic

strategies require complete and accurate analysis of the neoformed vascular network. However, methods of assessment, such as histology with confocal or two-photon microscopy, laser Doppler, microangiography, fluorescent microspheres, magnetic resonance angiography, positron emission tomography, are not always precise or quantitative; they focus on a limited area of study, reveal capillary density primarily in 2 dimensions, and represent superficial blood flow[137]. Currently, micro-computed tomography (MicroCT) is the only structural imaging modality that provides a high-resolution volumetric representation of vascular structures that directly reflects the level of development of neo-vasculature. After the injection of a radiopaque contrast agent, MicroCT can image the vascular network in 3D in an entire organ [134, 135] and give quantitative data. In combination with functional information from other imaging modalities such as MicroPET, ultrasound, or microscopy, MicroCT has the potential to advance angiogenesis related research even further[138].

In our study, MicroCT imaging revealed smaller and fewer arterial collaterals in $Gja5^{-/-}$ mice compared to $Gja5^{+/+}$ mice. Such differences may well account for the perfusion deficit observed in $Gja5^{-/-}$ mice. The smaller collateral arterial diameters in $Gja5^{-/-}$ mice after FAO pointed to a potential defect in arteriogenesis compared to $Gja5^{+/+}$ mice. But the diameters of pre-existing collateral arteries in left hindlimb did not differ between $Gja5^{-/-}$ mice and $Gja5^{+/+}$ mice. So we think that Gja5 plays a functional role in arteriogenesis, and MicroCT is one of the most promising imaging modalities for vascular exploration. Its extremely high spatial resolution presents a unique opportunity for studying the structure, organization, and, to some extent, even the function of blood vessels[138].

3. Gja5 conditional mutant mice had a small perfusion reduction at 3 days after FAO.

Since it can be argued that reduced perfusion in $Gja5^{-/-}$ mice may also result from the loss of Gja5 mediated vasodilatory responses, we have now taken advantage of the Cre-LoxP system to create Gja5-conditional mutant mice in which Cx40 is deleted only from the endothelium.

Endothelial specific deletion of Gja5 before FAO resulted in a small perfusion reduction at 3 days after FAO, but perfusion was restored to normal afterwards. This corresponds to a time point at which Gja5 expression is high in the ischemic hindlimb in $Gja5^{+/+}$ mice. An experimental limitation of our tamoxifen inducible Tie-2 Cre system is that the Cre recombination did not completely inhibit Gja5 expression, and that residual Gja5 protein function may have accounted for the relatively normal perfusion levels. However, in summary, our data support a model in which impaired flow-induced outward remodeling reduced the number of collateral vessels and there was no relationship between vasodilation mediated by loss of Gja5 and the perfusion deficit in $Gja5^{-/-}$ mice.

4. Reduced Gja5 in endothelial cells may play a functional role in arteriogenesis.

FAO induced Gja5 expression in the ischemic hindlimb to a similar extent in $Gja5^{+/-}$ mice and $Gja5^{+/+}$ mice. At 3 days after occlusion, expression was elevated 4-fold in the ischemic hindlimb, both in $Gja5^{+/-}$ mice and $Gja5^{+/+}$ mice. Expression levels returned to baseline values at 7 days after occlusion. Interestingly, Pipp et al.[44], demonstrated the importance of FSS in arteriogenesis by means of a porcine ischemic hindlimb model with extremely high levels of collateral flow and FSS. Normally, during the later phases of arteriogenesis, FSS decreases as the collateral diameter increases so that FSS normalizes. This drop in FSS acts as a signal to arrest proliferation and as a result, prevents further collateral growth before an optimal adaptation is reached. Pipp and colleagues [44] demonstrated that sustained, elevated FSS in their arteriovenous shunt model further significantly increased the size of collaterals, thus establishing that FSS is a dominant morphogenic factor in collateral growth. From above, we can see that the tendency for the change of Gja5 expression after femoral artery occlusion is the same as the change of FSS in arteriogenesis. Eno Essien Ebong et al.[139] believe that fluid shear stress regulates specific connexin composition and functional state of vascular gap junctions, potentially determining levels and type of communication and possibly cellular phenotype. So we believe that Gja5 plays a functional role in arteriogenesis, but more research needs to be done into the relationship between Gja5 expression and

FSS after femoral artery occlusion.

Gja5 expressed in arterial endothelium mediates vasodilatory responses. To examine the involvement of this functional component, we examined mice in which we deleted Gja5 expression specifically from the endothelium before FAO. Expression of Gja5 was measured in the femoral artery and gastrocnemius muscle. Reduced expression of Gja5 after endothelial specific deletion of Gja5 showed that Gja5 plays a functional role in arteriogenesis.

Altogether, we postulate that Gja5 may function as a positive modulator in arteriogenesis and serve as an arterial marker.

CHAPTER FIVE: SUMMARY

Background

Cardiovascular diseases are the number one cause of death globally[104]. Arteries are the key vessels affected in cardiovascular diseases and the study of mechanisms of arterial growth and repair are, therefore, of fundamental interest. Arterial and venous vascular networks show a distinct genetic signature, function and branching architecture[105, 106]. Specification of arterial-venous vessel identity and formation of branched vascular networks occur during early embryogenesis and are modulated by hemodynamic factors[107-109], but the precise mechanisms are unclear. Blood circulation creates mechanical forces in vessels[28, 110] and affects oxygenation of developing organs.

At present neuropilin-1[111, 112], Unc5b[116], Notch1, Notch4, Jag1, Jag2 and Dll4 [117-121], are established arterial markers. In the mouse, hemodynamic factors contribute to arterial-venous differentiation, involving regulation of arterial marker expression[108, 109]. In the adult, hemodynamic factors regulate the enlargement and outgrowth of collateral arterial networks upon arterial stenosis[3, 11]. Several molecules originally described in embryonic arterial remodeling, also modulate the efficiency of arterial collateralization in the adult[12].

Besides these classical morphogenes, arteries also express the gap-junctional proteins Gja4 (Connexin-37) and Gja5 (Connexin-40)[69, 122-124]. Gap junctional proteins mediate direct diffusion of signals between adjacent cells[125]. In the microcirculation, gap junction proteins facilitate electrical coupling between endothelial cells[41], which plays an important role in the regulation of vascular tone[59, 78], particularly in response to changes in tissue metabolic demand. The mechanism underlying arterial specific regulation of connexins is yet unknown.

Objectives

We hypothesized that arterial Gja5 expression plays a functional role in flow driven

arteriogenesis.

Methods

In mice, Gja5 (Connexin-40) was expressed in arteries. Although the participation of the gap junction in vascular function seems to be very complex, the development of connexin-knockout animals has been a great contribution to our understanding of how these proteins work in the vasculature. We made use of different kinds of mice for Connexin-40 mutant ($Gja5^{-/-}$), Connexin-40 floxed (Gja5f/f), and tamoxifen inducible Tie-2 Cre (Tie2CreERT2). Gja5 expressed in arterial endothelium mediates vasodilatory responses. To study the involvement of this functional component, we examined mice in which we deleted Gja5 expression specifically from the endothelium before FAO. We also set up flow driven arteriogenesis model: the hindlimb femoral artery occlusion model. In mice, ligation of the femoral artery results in the flow driven formation of a collateral arterial network that bypasses the occlusion**.** This process results in restoration of perfusion to the ischemic regions. The efficacy of this process can be assessed with repetitive non-invasive evaluation of hindlimb perfusion using laser-doppler flow imaging (LDF). The efficacy of blood transport into the compromised hindlimb depends on the size and number of the collateral arteries. To address this, we perfused the hindlimbs of $Gja5^{-/-}$ mutant and $Gja5^{+/+}$ mice with contrast agent (Microfil) under maximal vasodilatory conditions, and imaged the morphology of the collateral arterial network at 7 days after FAO using MicroCT. To image vessels below the detection limit of the MicroCT, we used histological analysis of the upper calf muscle.

Results

We observed significantly smaller lumen diameters in $Gja5^{-/-}$ mice when compared to $Gja5^{+/+}$ mice. We also obtained genetic evidence in mice showing the functional importance of Gja5 in flow driven arterial remodeling and collateral arterial network development.

Conclusion

Our data support a model in which impaired flow-induced outward remodeling reduced the number of collaterals and there was no relationship between vasodilation mediated by loss of Gja5 and the perfusion deficit in $Gja5^{-/-}$ mutant mice.Genetic deletion of Gja5 function in mice resulted in reduced arteriogenesis in femoral artery occlusion models. Gja5 plays a functional role in flow driven arteriogenesis.

ZUSAMMENFASSUNG

Hintergrund

Herz-Kreislauf Erkrankungen sind die häufigste Todesursache weltweit. Hauptsächlich sind Arterien bei Herz-Kreislauf Erkrankungen betroffen, deren Untersuchung daher von grundlegendem Interesse ist. Arterielle und venöse Netzwerke weisen eine unterschiedliche genetische Signatur, Funktion und Verzweigungsmuster auf. Diese Spezifikation der arterio-venösen Gefäßidentität und die Bildung der verzweigten Gefäßnetzwerke treten während der Embryogenese auf und werden durch hämodynamische Faktoren moduliert. Die genauen Mechanismen sind bisher aber ungeklärt. Durch die Zirkulation des Blutes werden mechanische Kräfte auf die Gefäße ausgeübt, welche die Sauerstoffversorgung der entwickelnden Organe beeinflussen.

In der Maus tragen hämodynamische Faktoren zur arteriellen-venösen Differenzierung bei, wie z.B. die Regulierung der Expression von arteriellen Markern. Derzeit sind Neuropilin-1, Unc5b, Notch1, Notch4, Jag1, Jag2 und Dll4 als arterielle Marker etabliert. In der adulten Maus wirken sich hämodynamische Faktoren auf die Vergrößerung des kollateralen arteriellen Netzwerkes nach arterieller Stenose aus. Bisher wurden einige Moleküle, die ursprünglich für den embryonalen arteriellen Umbau beschrieben worden sind, auch für die Modellierung der arteriellen Kollateralbildung im adulten Tier entdeckt.

Neben den klassischen Morphogenen exprimieren Arterien die Gap-Junction Proteine Gja4 (Connexin-37) und Gja5 (Connexin-40). Gap Junction Proteine sind kanalbildende Proteinkomplexe, welche den Transport von Molekülen zwischen benachbarten Zellen direkt vermitteln. In der Mikrozirkulation ermöglichen Gap Junction Proteine die elektrische Kommunikation zwischen Endothelialzellen, die für den vaskulären Tonus eine wichtige Rolle spielen. Der zugrunde liegende Mechanismus der arteriellen Regulierung der Connexine ist aber noch unbekannt.

Ziel

Es wird vermutet, dass die arterielle Expression von Gja5 eine funktionale Rolle in der flow-driven Arteriogenese spielt.

Methoden

Mit Hilfe der Entwicklung der Connexin knockout (KO) Mäuse wurde ein großer Beitrag zum Verständnis der Gefäßfunktion der Gap Junction Proteine geleistet. Wir nutzten verschiedene Connexin KO Modelle: Connexin-40 (Gja5-/-), Connexin-40 floxed (Gja5f/f) und Tamoxifen induzierbare Tie-2 Cre Maus (Tie2CreERT2). Das im arteriellen Endothelium lokalisierte Gap Junction Protein Gja5 vermittelt vasodilatorische Signale. Zur Untersuchung von Gja5 entwickelten wir ein Modell für flow driven Arteriogenese: hindlimb femoral artery Okklusion (FAO). Um die Funktion von Gja5 näher zu analysieren, verwendeten wir Mäuse in denen die Expression von Gja5 spezifisch im Endothelium vor der FAO deletiert wurde. Die Okklusion der femoralen Arterie resultiert in der Bildung eines kollateralen arteriellen Netzwerkes, welches die Okklusion umgeht. Dieser Prozess führt zur Perfusion der ischämischen Region, dessen Effizienz mit einem Laser-Doppler-Fluß-Bildanalysator (LDF) bewertet werden kann. Die Effizienz des Bluttransports im Hinterlauf hängt von der Größe und Anzahl der Kollateralarterien ab. Unter vasodilatorischen Bedingungen perfundierten wir die Hinterläufe von Gja5-/- und Gja5+/+ Mäusen mit einem Kontrastmittel (Microfil) und bildeten die Morphologie der kollateralen arteriellen Netzwerke 7 Tage nach der FAO mit einem MicroCT ab. Die Kollateralen, welche nicht mit dem MircoCT erfasst werden konnten, wurden mit Hilfe histologischer Analysen bewertet.

Ergebnisse

Der Lumendurchmesser in den Gja5-/- Mäusen ist im Vergleich zu den Gja5+/+ Mäusen signifikant kleiner. Weiterhin konnten wir die funktionale Bedeutung von Gja5 in der flow driven Arteriogenese und der Entwicklung des kollateralen Netzwerkes auf genetischer Ebene zeigen.

Fazit

Die ermittelten Daten unterstützen ein Modell, indem durch Blutfluss induziertes beeinträchtigtes outward remodeling zu einer reduzierten Anzahl von Kollateralen führt. Genetische Deletion der Funktion von Gja5 in Mäusen führt zu verminderter Arteriogenese im FAO Modell. Gja5 übt eine funktionale Rolle in der flow driven Arteriogenese aus.

CHAPTER SIX: STUDY LIMITATIONS AND PERSPECTIVES

Arteriogenesis (collateral vessel growth) is triggered by fluid shear stress in case of an arterial occlusion. It is an important focus in current cardiovascular research as it might provide new therapeutic opportunities. But the molecular mechanisms underlying arteriogenesis are not yet completely understood. Arteries express the gap-junctional proteins Gja4 (Connexin-37) and Gja5 (Connexin-40)[69, 122-124]. Gap junctional proteins mediate direct diffusion of signals between adjacent cells[41, 125]. In the microcirculation, gap junction proteins facilitate electrical coupling between endothelial cells[41], which plays an important role in the regulation of vascular tone[78, 98], particularly in response to changes in tissue metabolic demand. In vessels, an increase in connexin expression may be implicated in the structural and functional alterations that take place after mechanical stretch, and could represent a compensatory response to the numerous insults associated with increased fluid pressure, possibly to control the elasticity of the vessel wall. The present study demonstrates that Gja5 may function as a positive modulator in arteriogenesis and serve as an arterial marker. But the mechanism underlying specific arterial regulation of connexins is yet unknown. In our study, our data support a model in which impaired flow-induced outward remodeling reduced the number of collaterals and there was no relationship between vasodilation mediated by loss of Gja5 and the perfusion deficit in $Gja5^{-/-}$ mutant mice., but an experimental limitation of our tamoxifen inducible Tie-2 Cre system is that the Cre recombination did not completely annihilate Gja5 expression, and that residual Gja5 protein function may have accounted for the relatively normal perfusion levels. So we will continue to do the experiment on specific endothelial ablation of Gja5 mice. We hypothesize that many more genes are involved in differential regulation of flow driven arteriogenesis, and that combining analysis on gene expression may aid to uncover the full repertoire of genes relevant to establish arterial function.

Cardiovascular diseases are the number one cause of death globally[104]. Although successful therapies exist to reduce plaque formation and restore blood flow in patients suffering from ischemic vascular diseases, there is still a significant portion of patients

who do not benefit from these treatment options. Identification of markers of collateral growth could help in determining patient prognosis and predicting therapy response and maybe even lead to new, proarteriogenic therapies. Development of preclinical animal models is needed to test these methods, as extreme caution needs to be taken when extrapolating research in mice to the clinical setting. This will also enable further investigation of mechanisms, e.g., signaling molecules involved in collateral artery growth, extending our knowledge, and possibilities in therapeutic application. Future research will therefore involve investigation of the mechanisms behind the individual response to arteriogenesis.

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CURRICULUM VITAE AND PUBLICATIONS

My career will not be published, for privacy reasons in the electronic version of my work.

STATEMENT IN LIEU OF OATH

I declare that the experiments described in the thesis were carried out by me.

Erklärung

Ich, [Haitao Wang], erkläre, dass ich die vorgelegte Dissertation mit dem Thema: [Arteriogenesis in Gja5 (Connexin-40) deficient mice] selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum 15.12.2010 Unterschrift Haitao Wang