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Modulation of vascular remodeling : a role for the immune system, growth factors, and transcriptional regulation

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Shear induced collateral artery growth modulated by endoglin, but not by ALK1

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Abstract

Objective: TGF-β stimulates both ischemia induced angiogenesis and shear stress induced arteriogenesis, by signaling through different receptors. How these receptors are involved in both these processes of blood flow recovery is not entirely clear. In this study the role of TGF-β receptors ALK1 and endoglin is assessed in neovascularization in mice.

Methods and Results: Unilateral femoral artery ligation was performed in mice heterozygous for either endoglin, or ALK1 and in controls. Blood flow recovery, monitored by laser Doppler perfusion imaging, was significantly hampered in both endoglin and ALK1 heterozygous mice compared to controls by maximal 40% and 49%, respectively. Collateral artery size was significantly reduced in endoglin heterozygous mice compared to controls, but not in ALK1 heterozygous mice. Capillary density in ischemic calf muscles was unaffected, but capillaries from endoglin and ALK1 heterozygous mice were significantly larger when compared to controls. To provide mechanistic evidence for the differential role of endoglin and ALK1 in shear induced or ischemia induced neovascularization, murine endothelial cells were exposed to shear stress *in vitro*. This induced increased levels of endoglin messengerRNA, but not ALK1.

Conclusions: In this study it is demonstrated that both endoglin and ALK1 play a crucial role in blood flow recovery. Importantly, endoglin is essential in both shear induced collateral artery growth and in ischemia induced angiogenesis, whereas ALK1 is only involved in ischemia induced angiogenesis.

Introduction

In human adult life neovascularization comprises two different processes, angiogenesis, in which ischemia induced sprouting of vessels leads to the formation of new capillaries, and arteriogenesis, where shear-stress-driven maturation of pre-existing collateral arteries results in formation of mature collateral arteries. Whereas angiogenesis is mainly ischemia driven, arteriogenesis is a highly inflammation driven process. Next to specific inflammatory cell subsets [1-5], various cytokines and growth factors contribute to arteriogenesis.

Therapeutic application of cytokines such as Monocyte Chemoattractant Protein (MCP-1) and growth factors such as basic Fibroblast Growth Factor (bFGF) and Vascular Endothelial Growth Factor (VEGF) stimulate arteriogenesis [6]. More recently, it was demonstrated that local delivery of Transforming Growth Factor-beta (TGF-β) also stimulates arteriogenesis [7, 8].

TGF-β is a multifunctional cytokine involved in the control of cell division, differentiation, migration, adhesion and programmed cell death. Furthermore, TGF-β is known to mediate inflammation and contributes to vascular remodeling, for example after vessel wall injury [9] and in stimulating blood flow recovery [7]. Moreover, TGF-β is also thought to play a crucial role in the regulation of angiogenesis, especially in tumor angiogenesis [10-12]. TGF-β signaling is initiated by the formation of a membrane complex of two TGF-β type II receptors and two TGF-β type I receptors, also termed activin receptor-like kinases or ALKs. This complex then initiates signalling responses into the cytoplasm by phosphorylating intracellular transcription factors called SMADs [13, 14].

In endothelial cells (ECs) TGF-β signaling is mediated by two TGF-β type I receptors, ALK5, which is also expressed in many other cell types, and ALK1, which is mainly expressed in ECs [15]. In addition, ECs express an accessory TGF-β type III receptor named endoglin (Eng), which is also expressed by vascular smooth muscle cells [16]. Vessel remodeling, EC proliferation and migration are inhibited by TGF-β signaling via TβRII/ALK5, which mediates SMAD2/3 phosphorylation [10, 17]. On the other hand these processes are stimulated in ECs by TGF-β signaling via an Endoglin/TβRII/ALK1 complex, which induces SMAD1/5 activation [18, 19]. Endoglin promotes the TGF-β / ALK1 signal transduction pathway [17] and it is assumed that ALK1 can indirectly inhibit TGF-β/ALK5 signaling [12].

Both ALK1 and endoglin modulate neovascularization by TGF-β mediated induction of blood flow recovery. Shear stress is an essential factor in neovascularization and has been demonstrated to play a role in activation of ALK5/TGF-β signaling in the endothelium [20, 21]. Furthermore, these hemodynamic changes are suggested to induce ALK1 expression in endothelial cells [22] and an active role for both endoglin and ALK1

is suggested in shear stress driven vascular adaptation [23, 24]. This suggests that an increase in shear stress during arteriogenesis might modulate the expression of ALK1 and endoglin. Endoglin is involved in the production of TGF-β and VEGF by ECs, the modulation of EC proliferation and differentiation, and recruitment and differentiation of vascular smooth muscle cells (SMCs) [25, 26]. After myocardial infarction endoglin is up regulated in angiogenic vessels from human and murine hearts [27]. ALK1 also plays an important role in EC proliferation [19] and migration and stabilization of vessel structure integrity by stimulating recruitment and differentiation of SMCs [22]. In humans deregulated TGF-β signaling by haplo-insufficiency for either endoglin or ALK1 results in arteriovenous malformations that are a hallmark of hereditary haemorrhagic telangiectasia type 1, and type 2 (HHT1 or -2) [19].
The individual role of endoglin and ALK1 in vessel growth is well described, it is yet unknown how these receptors are involved in blood flow recovery after induction of hind limb ischemia. Jerkic et al [26] have studied the role of haplo-insufficiency for endoglin after induction of ischemia, but the analysis was restricted to the neovascularization in the adductor thigh muscle only. Capillary formation in the ischemic gastrocnemius muscle was not studied yet. This is however quite important especially in comparison to the effect of ALK1 haplo-insufficiency.
In the present study we investigate the role of endoglin and ALK1 in both shear induced collateral artery growth and ischemia induced angiogenesis. Induction of hind limb ischemia in mice heterozygous for either endoglin or ALK1 is a good model to study the involvement of these two receptors in neovascularisation.

Methods

Mice

All animal experiments were approved by the committee on animal welfare of our institute. For all experiments male mice were used, aged 12-16 weeks. Endoglin (C57BL/6 background) heterozygous mice (Eng+/-) [28] and ALK1 (C57BL/6) heterozygous mice (ALK1+/-) [19] and corresponding littermates (wild type) were bred in our institute. All animals were fed regular chow diet (Sniff Spezialdiäten GMBH, Soest, Germany).

Surgical procedure and analysis of arteriogenesis

Surgical induction of hind limb ischemia in the mice, as well as analysis of collateral formation by either angiography or laser-doppler-perfusion-imaging (LDPI; Moor Ltd, Axminster, United Kingdom), and tissue collection was performed as described [4, 29].

Angiography

To study collateral vessel development, post-mortem angiograms of both hind limbs were made using polyacrylamide-bismuth contrast mixture (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands) 7 days after femoral artery occlusion, as described [29]. Grading of collateral filling was performed in a single blinded fashion by two independent observers and was based on the Rentrop score. Grading was as follows: 0 = no filling of collaterals, 1 = filling of collaterals only, 2 = partial filling of distal femoral artery, 3 = complete filling of distal femoral artery.

Immunohistochemistry

Muscles were dissected upon sacrifice of the mice. Gastrocnemius and adductor muscle of both hind limbs were dissected and were overnight formalin-fixated. 5 μm thick paraffin-embedded cross sections were stained using antibodies for PECAM-1 (CD31) (BD Biosciences, San Jose, United States of America), α-Smooth Muscle (αSM)-actin (Clone1A4) (DAKO, Glostrup, Denmark) or Erythroid lineage TER-119 (Santa Cruz Biotechnology, Santa Cruz, United States of America) for erythrocyte staining. Staining for both CD31 and αSM-actin were quantified from sections photographed randomly (3 images per section) using image analysis (Qwin, Leica, Wetzlar, Germany).

Endothelial cell culture

Mouse embryonic endothelial cell (MEEC) isolation was previously described [30]. Cells were passaged twice a week and maintained on 1% w/v gelatin (Merck, Darmstadt, Germany) in DMEM medium (Invitrogen, Breda, the Netherlands) supplemented with 4.5 g/L D-glucose (Invitrogen), 25 mM Hepes (Invitrogen), 110 mg/L sodium pyruvate (Invitrogen), 10% (v/v) heat inactivated Fetal Calf Serum (Sigma-Aldrich Chemie, Steinheim, Germany), 1% (v/v) antibiotic/ antimycotic solution (Invitrogen), and 2mM Lglutamine (Invitrogen). For the shear stress experiments, MEEC were seeded on fixed 1% (w/v) gelatin coated coverslips and grown to confluence. EC were subjected to 0.5 Pa or -5 dyn/cm2 shear stress for 24 hours at 37°C and 5% CO2 in a re-circulation parallel plate flow system as previously reported [21]. Responses of shear-exposed cells were compared to those of static cultures. Directly following exposure to flow cells were lysed for RNA isolation.

mRNA analysis Q-PCR

Total RNA was isolated (RNeasy, Qiagen Benelux, Venlo, The Netherlands) and was treated with DNAse-I (Qiagen) according to the manufacturer's protocol. IScript cDNA synthesis kit (Bio-Rad, Veenendaal, The Netherlands) was used to reverse transcribe 500ng of RNA into cDNA. Real-time Q-PCR was performed using iQ SYBR Green

Supermix (Bio-rad) in a Mx3000 real-time thermocycler (Stratagene, Santa Clara, United States of America) as described [31]. The reaction mixture consisted of the following: 1x PCR Master Mix, 1μl cDNA template, and 10 pmol of each specific primer. Dissociation analysis was performed in all reactions to exclude the presence of primer-dimers and confirm the amplification of unique targets. No template controls were used as negative controls. Relative expression levels were normalized to the housekeeping gene BACT to compensate for the differences in RNA input.

Statistical analysis

Results are expressed as mean±SEM. Comparisons between medians were performed using the Mann Whitney or Wilcoxon test as appropriate. Ordinal scores (e.g. angiography) were compared using Pearson Chi-Square test. A p-value <0.05 was considered statistically significant.

Results

Impaired blood flow recovery and reduced collateral artery size in Eng+/- mice

After induction of hind limb ischemia by femoral artery ligation Eng+/- mice revealed a significant impairment of blood flow recovery when compared to controls. The blood flow recovery was significantly reduced by 40% after 3 days, 33% after 7 days, and by 16% after 21 days (p-value=0.05). Eng+/- mice reached a maximum paw perfusion ratio at 28 days of 0.73±0.06 (figure 1).

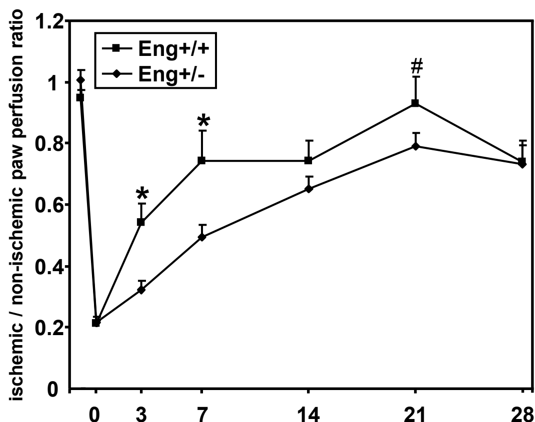

Figure 1. Ischemic / non-ischemic paw perfusion ratios of endoglin heterozygous (Eng+/-, n=10) and control mice, measured by LDPI over 28 days (n=9; originally 10, but 1 mice died due to an unrelated cause of death and was therefore not included in the analysis). (*p value <0.05), (# p value =0.05)

For performing post-mortem angiography to demonstrate presence of collateral arteries in adductor thigh muscle, Eng+/- mice (n=10) and controls (n=10) were sacrificed 7 days after femoral artery ligation). LDPI follow-up until 7 days showed significant impaired blood flow recovery in Eng+/- mice when compared to controls at 3 and 7 days after ligation (figure 2A-B), similarly as was observed in the long term follow up experiment demonstrated in figure 1. As shown by representative images (figure 2C), no obvious differences in the presence of collaterals between Eng+/- mice and controls could be observed.

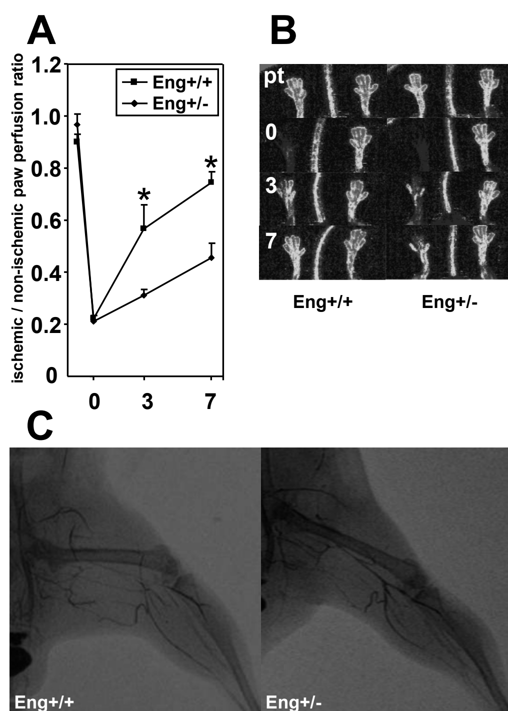

Figure 2. A: Ischemic / non-ischemic paw perfusion ratios of Eng+/- (n=9; originally 10, but 1 mice died due to an unrelated cause of death and was therefore not included in the analysis) and control mice (n=8; originally 10, but 2 mice died due to an unrelated cause of death and were therefore not included in the analysis), measured by LDPI over 7 days. (*p value <0.01) **B:** Representatives of Laser Doppler Perfusion Imaging (LDPI) images of Eng+/- and control mice that were used to obtain post-mortem angiographic images. Several time points are displayed: pre-treatment (pt), t=0, t=3 and t=7. **C:** Representative angiographic images of upper hind limb of Eng+/- and control mice 7 days after femoral artery ligation. *See color figure on page 236.*

In the search for an explanation for the impaired blood flow recovery, we determined whether collateral artery size was affected in Eng+/- mice, and performed an α-smooth muscle (αSM)-actin staining on adductor thigh muscles collected 7 days after ligation.

The analysis for collateral artery size revealed significant smaller collateral arteries in Eng+/- mice when compared to controls, monitored as collateral artery surface on cross sections (figure 3A). Collateral artery size in adductor thigh muscles collected 28 days after ligation was not different between Eng+/- and control mice (figure 3B).

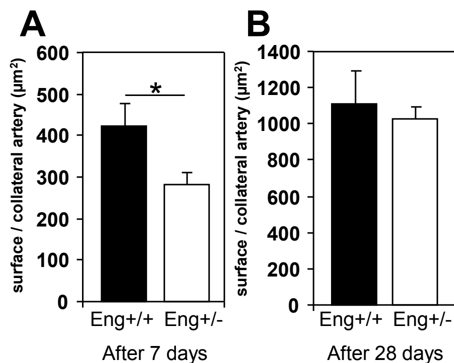

Figure 3. Analysis for collateral artery size, measured as surface (μm^2) per collateral artery in left adductor thigh muscles of Eng+/- and control mice (n=6-10) **A:** 7 days and **B:** 28 days after femoral artery ligation. (* p value <0.05)

Enlarged capillaries in Eng+/- mice after induction of ischemia

In order to analyze whether endoglin deficiency also affected the angiogenic response in the ischemic hind limb , the calf muscles (gastrocnemius muscle) were analyzed, and quantification of angiogenesis was performed by analyzing the capillaries formed in challenged (left) and unchallenged (right) calf muscles 7 days and 28 days after ligation (PECAM-1 staining). As depicted in figure 4A, analysis for capillary size demonstrated significant larger capillaries in Eng+/- mice than observed in controls 7 days after ligation. In both Eng+/- and control mice a significant increase in capillary density is observed, 2.2 and 2.1 fold increase respectively, when compared to capillary density in right calf muscles. No difference in capillary density was observed between both groups (figure 4B).

Analysis for capillary size in calf muscles collected 28 days after ligation revealed a reduction in capillary size in the challenged left calf muscles of Eng+/- mice compared to the capillary size observed at day 7. After 28 days capillary size was not different anymore between Eng+/- mice and controls (figure 4E). A similar pattern was observed for capillary density, since capillary density in challenged left calf muscles of both Eng+/- mice and controls was decreased when compared to capillary densities observed 7 days after femoral artery ligation in left calf muscles of both Eng+/- mice and controls. As depicted in figure 4F, this decrease was less pronounced in Eng+/- mice that displayed significant higher capillary densities in left calf muscles when compared to controls.

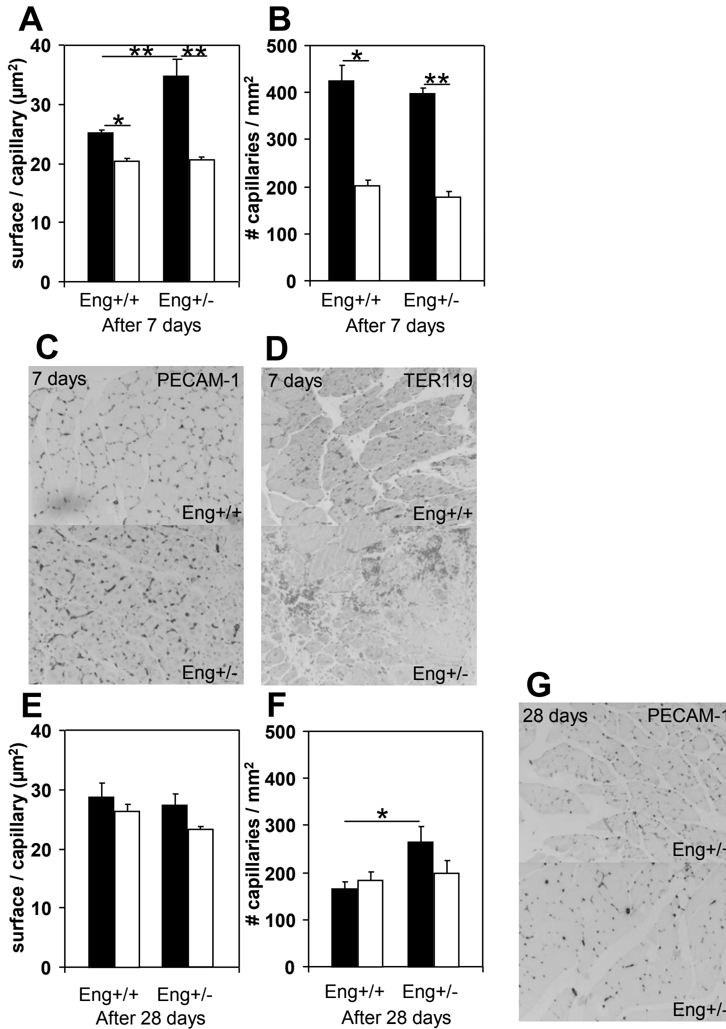

Figure 4. Quantification of ischemia induced angiogenesis by analysis of PECAM-1 stained left (ligated-side) and right (control-leg) calf muscles of Eng+/- and control mice at 7 and 28 days after femoral artery ligation. Closed bars represent left calf muscles, open bars represent right calf muscles. (* p value <0.05, ** p value <0.01)
A: Capillary size, measured as surface per individual capillary (μm^2), 7 days after femoral artery ligation (n=8-9 per group). **B:** Capillary density, measured as number of capillaries per mm^2, 7 days after femoral artery ligation. (n=8-9 per group). **C:** Representative pictures (magnification 20x) of PECAM-1 stained ischemic calf muscles of Eng+/- and control mice at 7 days after femoral artery ligation. **D:** Representative pictures (magnification 20x) of TER-119 staining (erythrocytes) demonstrating evasion of erythrocytes outside of the capillary vasculature into the interstitial tissue compartment in Eng+/- ischemic calf muscles when compared to ischemic calf muscles of control mice 7 days after femoral artery ligation. **E:** Capillary size, measured as surface per individual capillary (μm^2), 28 days after femoral artery ligation (n=9-10 per group). **F:** Capillary density, measured as number of capillaries per mm^2, 28 days after femoral artery ligation (n=9-10 per group). **G:** Representative pictures (magnification 20x) of PECAM-1 stained ischemic calf muscles of Eng+/- and control mice at 28 days after femoral artery ligation. *See color figure on page 237.*

Impaired blood flow recovery in ALK1+/- mice

After induction of hind limb ischemia by femoral artery ligation in ALK1+/- mice, blood flow recovery was significantly impaired when compared to controls. Directly after femoral artery ligation blood flow recovery in ALK1+/- mice was significantly reduced by 20%, and was also significantly reduced after 3, 7 and 21 days by 49%, 33% and 17% respectively (figure 5). Despite impaired blood flow recovery ALK1+/- mice reached a paw perfusion ratio of 0.98±0.07 at 28 days which was equal to that observed in control mice.

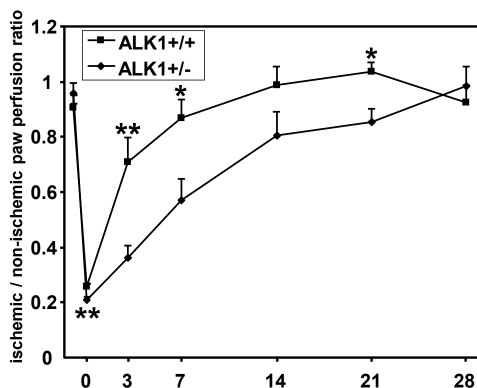

Figure 5. Ischemic / non-ischemic paw perfusion ratios of ALK1 heterozygous (ALK+/-, n=10) and control mice, measured by LDPI over 28 days (n=9; originally 10, but 1 mice died due to an unrelated cause of death and was therefore not included in the analysis.). (* p value <0.05, ** p value <0.01)

To demonstrate the presence of collateral arteries in the adductor thigh muscle, post-mortem angiograms were made 7 days after femoral artery ligation in ALK1+/- mice (n=10) and controls (n=10). As depicted in figures 6A and B, LDPI follow-up again demonstrated impaired blood flow recovery in ALK1+/- mice at both 3 and 7 days after femoral artery ligation, similarly as was observed previously (figure 5). As shown in representative angiograms (figure 6C) no prominent differences in collateral artery presence between ALK1+/- and control mice were observed.

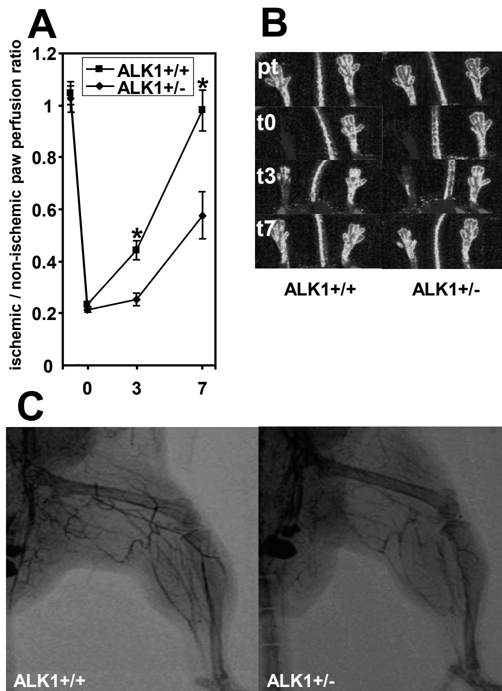

Figure 6. A: Ischemic / non-ischemic paw perfusion ratios of ALK1+/-Eng+/- (n=10) and control mice (n=10). (* p value <0.01), measured by LDPI over 7 days. **B:** Representatives of Laser Doppler Perfusion Imaging (LDPI) images of Eng+/- and control mice that were used to obtain post-mortem angiographic images. Several time points are displayed: pre-treatment (pt), t=0, t=3 and t=7. **C:** Representative angiographic images of upper hind limb of ALK1+/- and control mice 7 days after femoral artery ligation. *See color figure on page 238.*

The impaired flow recovery in the ALK1+/- mice may also be due to differences in the diameter of the newly formed collaterals. Therefore the collateral artery size was quantified in ALK1+/- mice by performing an αSM-actin staining for smooth muscle cells surrounding the newly formed collaterals in the adductor thigh muscles collected 7 days after ligation. This analysis revealed no significant differences in collateral artery size between ALK1+/- mice when compared to controls (figure 7A). Additional analysis for collateral artery size on adductor thigh muscles collected 28 days after ligation, also did not reveal differences in collateral artery size between ALK1+/- and controls (figure 7B).

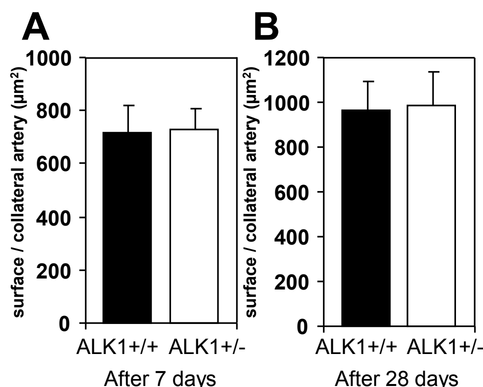

Figure 7. Analysis for collateral artery size, measured as surface (μm^2) per collateral artery in left adductor thigh muscles of ALK1+/- and control mice (n=6-10) **A:** 7 days and **B:** 28 days after femoral artery ligation.

ALK1+/- mice have larger capillaries after induction of ischemia

The impaired blood flow recovery in ALK1+/- mice could be explained by the newly formed capillary bed in the calf musculature due to differences in the angiogenesis response. Therefore the angiogenic response was quantified by performing a staining for endothelial cell (using an anti-PECAM-1 antibody) on tissue samples of challenged (left) and unchallenged (right) calf muscles of ALK1+/- and control mice collected 7 and 28 days after femoral artery ligation. Remarkably, analysis for capillary size revealed significant larger capillaries in ALK1+/- mice compared to controls 7 days after ligation (figure 8A). As depicted in figure 8B, our analysis for capillary density did not reveal differences between ALK1+/- and control mice 7 days after ligation. Induction of angiogenesis was normal in ALK1+/- mice, since a similar significant increase in capillary density was observed in left calf muscles compared to capillary density in right calf muscles, 1.8 and 1.9 fold increase respectively.

At 28 days the average capillary size in left calf muscles of ALK1+/- mice was slightly decreased and not different anymore from capillary size of controls (figure 8E). At this time point, the capillary density in both groups was not further enhanced (figure 8F). These observations correspond to the LDPI blood flow recovery data, since no differences in blood flow recovery existed 28 days after femoral artery ligation.

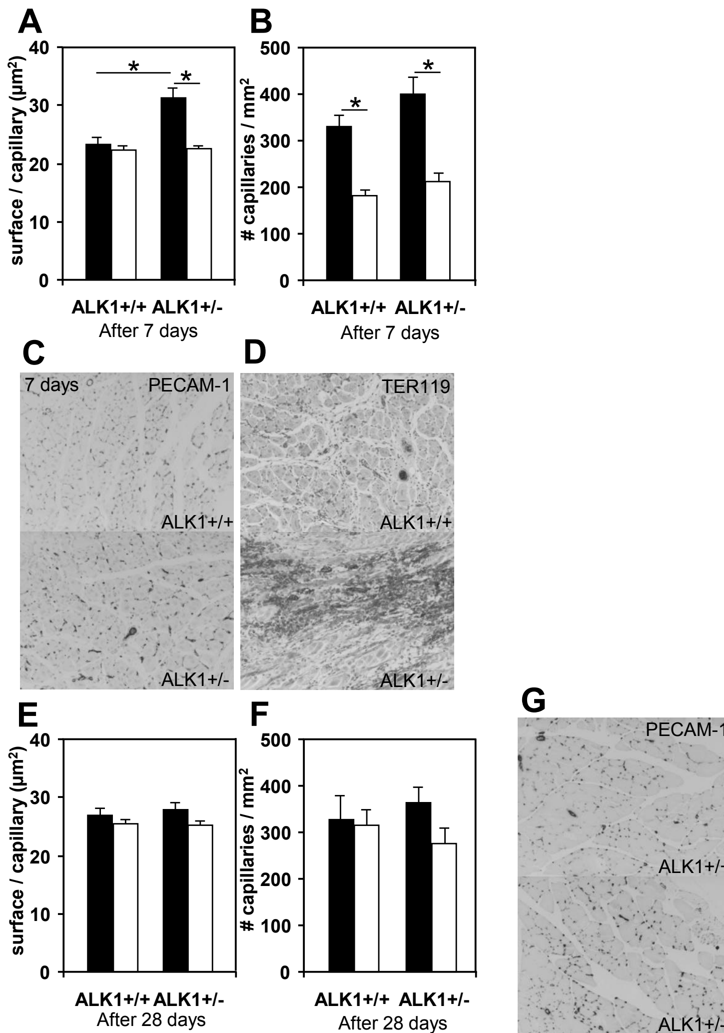

Figure 8. Quantification of ischemia induced angiogenesis by analysis of PECAM-1 stained left (ligated-side) and right (control-leg) calf muscles of ALK+/- and control mice at 7 and 28 days after femoral artery ligation. Closed bars represent left calf muscles, open bars represent right calf muscles. (* p value <0.01)

A: Capillary size, measured as surface per individual capillary (μm^2), 7 days after femoral artery ligation (n=10 per group). **B:** Capillary density, measured as number of capillaries per mm^2, 7 days after femoral artery ligation. (n=10 per group). **C:** Representative pictures (magnification 20x) of PECAM-1 stained ischemic calf muscles of ALK1+/- and control mice at 7 days after femoral artery ligation. **D:** Representative pictures (magnification 20x) of TER-119 staining (erythrocytes) demonstrating substantial evasion of erythrocytes outside of the capillary vasculature into the interstitial tissue compartment in ALK1+/- ischemic calf muscles when compared to ischemic calf muscles of control mice 7 days after femoral artery ligation. **E:** Capillary size, measured as surface per individual capillary (μm^2), 28 days after femoral artery ligation (n=9-10 per group). **F:** Capillary density, measured as number of capillaries per mm^2, 28 days after femoral artery ligation (n=9-10 per group). **G:** Representative pictures (magnification 20x) of PECAM-1 stained ischemic calf muscles of ALK1+/- and control mice at 28 days after femoral artery ligation. *See color figure on page 239.*

Shear stress induced up regulation of endoglin but not of ALK1 mRNA

The results of these experiments show a striking difference in response in the Eng+/- and ALK1+/- mice with regard to the arteriogenic response. Since collateral formation is a shear stress induced process, the observed differences might be due to different responses of endoglin and ALK1 to shear stress. To study this hypothesis in more detail murine embryonic endothelial cells (MEEC) were cultured under condition of flow, to mimic shear stress *in vitro*, for a period of 24 hours and subsequently the cells were harvested and the expression of endoglin and ALK1 was analyzed at the mRNA level. As can be observed in figure 9, no differences in ALK1 mRNA expression in MEEC were observed under flow conditions, whereas a significant 50% increase in endoglin mRNA expression was observed in the MEEC exposed to a flow of 5 dyn/cm^2 for 24 hours. These data clearly demonstrate a difference in shear stress sensitivity for endoglin and ALK1 expression in endothelial cells.

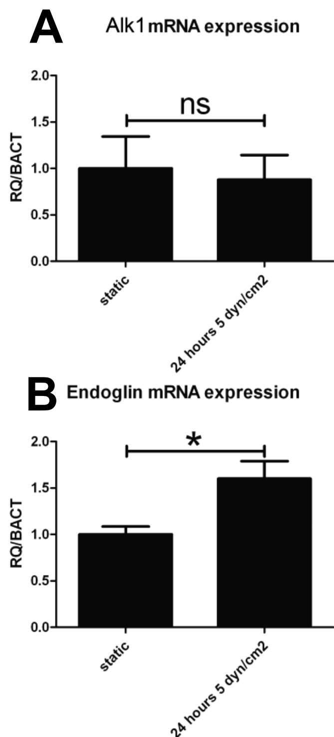

Figure 9. Shear induced regulation of endoglin and ALK1 expression in Mouse Embryonic Endothelial Cells (MEEC). Real-time Q-PCR determined messenger RNA levels, normalized for BACT (n=4), for endoglin (**A**) or ALK1 (**B**) in MEEC under static condition or exposed to shear stress (0.5 Pa or 5 dyn/cm^2) for 24 hours. (* p value <0.05)

Discussion

In the present study we addressed the role of TGF-β signaling receptors endoglin and ALK1 in blood flow recovery after induction of acute hind limb ischemia in mice. We demonstrated that haplo-insufficiency for endoglin affects both shear induced collateral artery growth and ischemia induced capillary angiogenesis, whereas haplo-insufficiency for ALK1 only resulted in disturbed capillary angiogenesis by the formation of dysplastic capillaries. *In vitro* data of mouse embryonic endothelial cells exposed to shear stress, confirms this additional contributory role of endoglin in shear stress induced collateral artery growth, as increased levels of endoglin messenger RNA (mRNA), but not of ALK1, were detected in these cells.

A previous study [26] demonstrated reduced vascularity in adductor thigh muscles of endoglin heterozygous mice after hind limb ischemia, which resembles to our observation of reduced vascularity in the present study. Moreover, in our hind limb ischemia model we studied blood flow recovery in both the upper- and lower hind limb, which allowed us to discriminate between shear induced collateral artery growth and ischemia induced capillary angiogenesis in the same mouse model. By this approach we were able to demonstrate that ALK1 and endoglin contribute differently to these two processes of blood flow recovery.

After hind limb ischemia endoglin heterozygous mice displayed significant reduced collateral artery size, indicating impaired arteriogenesis, when compared to littermate control mice. Furthermore, ischemia induced angiogenesis in the calf muscles of these mice resulted in disturbed capillary formation, represented by significant larger capillaries than in calf muscles of controls. Dysfunction of these capillaries was indicated by staining for erythrocytes, which demonstrated evasion of erythrocytes outside of the capillary vasculature into the interstitial tissue compartment. As capillary density was not affected in the calf muscles of endoglin heterozygous mice, this suggests that endothelial cell migration and reorganization, but not growth (proliferation), is the disturbed angiogenic component in these mice. This was also demonstrated by Mahmoud et al in conditional endoglin knockout mice [32]. The fact that both shear induced arteriogenesis and ischemia induced angiogenesis are affected in the endoglin heterozygous mice that we used in the present study, suits well with the fact that endoglin is expressed by both endothelial and vascular smooth muscle cells [16, 33] and by fibroblasts in perivascular stroma of arteries [24].

In ALK1 heterozygous mice blood flow recovery was found to be impaired by only defective angiogenesis, but not shear induced collateral artery growth, since these mice only displayed significant larger capillaries indicating capillary dysplasia, which is associated with ALK1 haplo-insufficiency [10, 12]. It has been described that ALK1

heterozygous mice stimulated with angiogenic growth factors have dysplastic and dilated capillaries and arteriovenous malformations [34]. Our hind limb ischemia model also induces the production of angiogenic growth factors [29], which explains significant increase of capillary size 7 days after ischemia. Endothelial cell proliferation was not affected in these mice, since a significant increase in capillary density, similar to controls, was observed. Here, analysis for erythrocytes outside capillary vessel walls also indicated that these dysplastic capillaries were indeed leaky and this implicates reduced functionality [10, 35], which should have had consequences for blood flow recovery in these mice.
The fact that in ALK1 heterozygous mice only ischemia induced angiogenesis is impaired, but not shear induced collateral artery growth, can be explained by the fact that ALK1 is mainly expressed in endothelial cells [15], but not, unlike endoglin [36], in vascular smooth muscle cells that play a role in collateral artery remodeling. Although ALK1 is suggested to promote SMC recruitment and differentiation, mainly based on observations in ALK1-null mice, haplo-insufficiency for ALK1 did not result in hampered collateral artery growth in our hind limb ischemia mouse model. An explanation could be that ALK1 heterozygosity has milder consequences than total depletion of ALK1. Besides this, ALK1 is demonstrated to limit nascent vessel caliber in reaction to shear stress, ALK1 haplo-insufficiency might therefore have facilitated normal collateral artery growth by reduced inhibition [23]. Finally, the observation that shear stress on cultured Mouse Embryonic Endothelial Cells (MEEC) induced high messenger RNA (mRNA) levels of endoglin, but not ALK1, provides a body of mechanistic evidence for this *in vivo* observation of impaired shear induced collateral artery formation in endoglin heterozygous mice, but not in ALK1 heterozygous mice.
Therefore we conclude that both TGF-β receptors endoglin and ALK1 play a contributory role in blood flow recovery. Importantly, our study for the first time demonstrates that endoglin is essential in both shear induced collateral artery growth and in ischemia induced capillary angiogenesis, whereas ALK1 is only involved in ischemia induced capillary angiogenesis.

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Disclosures
The authors confirm that there are no conflicts of interest.

References

1. Bergmann CE, Hoefer IE, Meder B, Roth H, van Royen N, Breit SM, Jost MM, Aharinejad S, Hartmann S, Buschmann IR. Arteriogenesis depends on circulating monocytes and macrophage accumulation and is severely depressed in op/op mice. *J Leukoc Biol* 2006 July;80(1):59-65.

2. Stabile E, Burnett MS, Watkins C, Kinnaird T, Bachis A, la Sala A., Miller JM, Shou M, Epstein SE, Fuchs S. Impaired arteriogenic response to acute hindlimb ischemia in CD4-knockout mice. *Circulation* 2003 July 15;108(2):205-10.

3. Stabile E, Kinnaird T, la Sala A., Hanson SK, Watkins C, Campia U, Shou M, Zbinden S, Fuchs S, Kornfeld H, Epstein SE, Burnett MS. CD8+ T lymphocytes regulate the arteriogenic response to ischemia by infiltrating the site of collateral vessel development and recruiting CD4+ mononuclear cells through the expression of interleukin-16. *Circulation* 2006 January 3;113(1):118-24.

4. van Weel V, Toes RE, Seghers L, Deckers MM, de Vries MR, Eilers PH, Sipkens J, Schepers A, Eefting D, van Hinsbergh VWM, van Bockel JH, Quax PHA. Natural killer cells and CD4+ T-cells modulate collateral artery development. *Arterioscler Thromb Vasc Biol* 2007 November;27(11):2310-8.

5. Zouggari Y, Ait-Oufella H, Waeckel L, Vilar J, Loinard C, Cochain C, Recalde A, Duriez M, Levy BI, Lutgens E, Mallat Z, Silvestre JS. Regulatory T cells modulate postischemic neovascularization. *Circulation* 2009 October 6;120(14):1415-25.

6. Voskuil M, van Royen N, Hoefer IE, Seidler R, Guth BD, Bode C, Schaper W, Piek JJ, Buschmann IR. Modulation of collateral artery growth in a porcine hindlimb ligation model using MCP-1. *Am J Physiol Heart Circ Physiol* 2003 April;284(4):H1422-H1428.

7. Grundmann S, van Royen N, Pasterkamp G, Gonzalez N, Tijsma EJ, Piek JJ, Hoefer IE. A new intra-arterial delivery platform for pro-arteriogenic compounds to stimulate collateral artery growth via transforming growth factor-beta1 release. *J Am Coll Cardiol* 2007 July 24;50(4):351-8.

8. van Royen N, Hoefer I, Buschmann I, Heil M, Kostin S, Deindl E, Vogel S, Korff T, Augustin H, Bode C, Piek JJ, Schaper W. Exogenous application of transforming growth factor beta 1 stimulates arteriogenesis in the peripheral circulation. *FASEB J* 2002 March;16(3):432-4.

9. Nabel EG, Shum L, Pompili VJ, Yang ZY, San H, Shu HB, Liptay S, Gold L, Gordon D, Derynck R, . Direct transfer of transforming growth factor beta 1 gene into arteries stimulates fibrocellular hyperplasia. *Proc Natl Acad Sci U S A* 1993 November 15;90(22):10759-63.

10. ten Dijke P, Arthur HM. Extracellular control of TGFbeta signalling in vascular development and disease. *Nat Rev Mol Cell Biol* 2007 November;8(11):857-69.

11. ten Dijke P, Goumans MJ, Pardali E. Endoglin in angiogenesis and vascular diseases. *Angiogenesis* 2008;11(1):79-89.

12. Pardali E, Goumans MJ, ten Dijke P. Signaling by members of the TGF-beta family in vascular morphogenesis and disease. *Trends Cell Biol* 2010 September;20(9):556-67.

13. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003 October 9;425(6958):577-84.

14. ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004 May;29(5):265-73.

15. Roelen BA, van Rooijen MA, Mummery CL. Expression of ALK-1, a type 1 serine/threonine kinase receptor, coincides with sites of vasculogenesis and angiogenesis in early mouse development. *Dev Dyn* 1997 August;209(4):418-30.

16. Mancini ML, Terzic A, Conley BA, Oxburgh LH, Nicola T, Vary CP. Endoglin plays distinct roles in vascular smooth muscle cell recruitment and regulation of arteriovenous identity during angiogenesis. *Dev Dyn* 2009 October;238(10):2479-93.

17. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J* 2002 April 2;21(7):1743-53.

18. Li B, Yin W, Hong X, Shi Y, Wang HS, Lin SF, Tang SB. Remodeling retinal neovascularization by ALK1 gene transfection in vitro. *Invest Ophthalmol Vis Sci* 2008 October;49(10):4553-60.

19. Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, Li E. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A* 2000 March 14;97(6):2626-31.

20. Egorova AD, Khedoe PP, Goumans MJ, Yoder BK, Nauli SM, ten DP, Poelmann RE, Hierck BP. Lack of Primary Cilia Primes Shear-Induced Endothelial-to-Mesenchymal Transition. *Circ Res* 2011 March 10.

21. Egorova AD, Van der Heiden K, van de Pas S, Venneman P, Poelma C, DeRuiter MC, Goumans MJ, Gittenberger-de Groot AC, ten Dijke P, Poelmann RE, Hierck BP. Tgfb/Alk5 signaling is required for shear stress induced Klf2 expression in embryonic endothelial cells. Developmental Dynamics. In press 2011.

22. Seki T, Yun J, Oh SP. Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. *Circ Res* 2003 October 3;93(7):682-9.

23. Corti P, Young S, Chen CY, Patrick MJ, Rochon ER, Pekkan K, Roman BL. Interaction between alk1 and blood flow in the development of arteriovenous malformations. *Development* 2011 April;138(8):1573-82.

24. Matsubara S, Bourdeau A, terBrugge KG, Wallace C, Letarte M. Analysis of endoglin expression in normal brain tissue and in cerebral arteriovenous malformations. *Stroke* 2000 November;31(11):2653-60.

25. Carvalho RL, Jonker L, Goumans MJ, Larsson J, Bouwman P, Karlsson S, ten Dijke P, Arthur HM, Mummery CL. Defective paracrine signalling by TGFbeta in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. *Development* 2004 December;131(24):6237-47.

26. Jerkic M, Rodriguez-Barbero A, Prieto M, Toporsian M, Pericacho M, Rivas-Elena JV, Obreo J, Wang A, Perez-Barriocanal F, Arevalo M, Bernabeu C, Letarte M, Lopez-Novoa JM. Reduced angiogenic responses in adult Endoglin heterozygous mice. *Cardiovasc Res* 2006 March 1;69(4):845-54.

27. Tian F, Zhou AX, Smits AM, Larsson E, Goumans MJ, Heldin CH, Boren J, Akyurek LM. Endothelial cells are activated during hypoxia via endoglin/ALK-1/SMAD1/5 signaling in vivo and in vitro. *Biochem Biophys Res Commun* 2010 February 12;392(3):283-8.

28. Arthur HM, Ure J, Smith AJ, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG. Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev Biol* 2000 January 1;217(1):42-53.

29. van Weel V, Deckers MM, Grimbergen JM, van Leuven KJ, Lardenoye JH, Schlingemann RO, van Nieuw Amerongen GP, van Bockel JH, van Hinsbergh VWM, Quax PHA. Vascular endothelial growth factor overexpression in ischemic skeletal muscle enhances myoglobin expression in vivo. *Circ Res* 2004 July 9;95(1):58-66.

30. Larsson J, Goumans MJ, Sjostrand LJ, van Rooijen MA, Ward D, Leveen P, Xu X, ten Dijke P, Mummery CL, Karlsson S. Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *EMBO J* 2001 April 2;20(7):1663-73.

31. Hierck BP, Molin DG, Boot MJ, Poelmann RE, Gittenberger-de Groot AC. A chicken model for DGCR6 as a modifier gene in the DiGeorge critical region. *Pediatr Res* 2004 September;56(3):440-8.

32. Mahmoud M, Allinson KR, Zhai Z, Oakenfull R, Ghandi P, Adams RH, Fruttiger M, Arthur HM. Pathogenesis of arteriovenous malformations in the absence of endoglin. *Circ Res* 2010 April 30;106(8):1425-33.

33. Adam PJ, Clesham GJ, Weissberg PL. Expression of endoglin mRNA and protein in human vascular smooth muscle cells. *Biochem Biophys Res Commun* 1998 June 9;247(1):33-7.

34. Hao Q, Su H, Marchuk DA, Rola R, Wang Y, Liu W, Young WL, Yang GY. Increased tissue perfusion promotes capillary dysplasia in the ALK1-deficient mouse brain following VEGF stimulation. *Am J Physiol Heart Circ Physiol* 2008 December;295(6):H2250-H2256.

35. Srinivasan S, Hanes MA, Dickens T, Porteous ME, Oh SP, Hale LP, Marchuk DA. A mouse model for hereditary hemorrhagic telangiectasia (HHT) type 2. *Hum Mol Genet* 2003 March 1;12(5):473-82.

36. Bot PT, Hoefer IE, Sluijter JP, van VP, Smits AM, Lebrin F, Moll F, de Vries JP, Doevendans P, Piek JJ, Pasterkamp G, Goumans MJ. Increased expression of the transforming growth factor-beta signaling pathway, endoglin, and early growth response-1 in stable plaques. *Stroke* 2009 February;40(2):439-47.