Actin-Binding Rho Activating Protein (*Abra*) Is Essential for Fluid Shear Stress–Induced Arteriogenesis

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***Objective*—**Arteriogenesis, the development of a collateral circulation, is important for tissue survival but remains functionally defective because of early normalization of fluid shear stress (FSS). Using a surgical model of chronically elevated FSS we showed that rabbits exhibited normal blood flow reserve after femoral artery ligature (FAL). Inhibition of the Rho pathway by Fasudil completely blocked the beneficial effect of FSS. In a genome-wide gene profiling we identified *actin-binding Rho activating protein* (*Abra*), which was highly upregulated in growing collaterals.

***Methods and Results*—**qRT-PCR and Western blot confirmed highly increased FSS-dependent expression of *Abra* in growing collaterals. NO blockage by L-NAME abolished FSS-generated *Abra* expression as well as the whole arteriogenic process. Cell culture studies demonstrated an Abra-triggered proliferation of smooth muscle cells through a mechanism that requires Rho signaling. Local intracollateral adenoviral overexpression of *Abra* improved collateral conductance by 60% in rabbits compared to the natural response after FAL. In contrast, targeted deletion of *Abra* in CL57BL/6 mice led to impaired arteriogenesis.

***Conclusions*—**FSS-induced *Abra* expression during arteriogenesis is triggered by NO and leads to stimulation of collateral growth by smooth muscle cell proliferation. **(*Arterioscler Thromb Vasc Biol*. 2009;29:2093-2101.)**

Key Words: arteriogenesis ■ *Abra* ■ fluid shear stress ■ smooth muscle cells ■ proliferation

The vascular system has the capacity to heal itself by the development and growth of a collateral circulation. In fact, the deleterious effects of arterial occlusions can be partially compensated, especially when the speed of occlusion is not acute but somewhat delayed. We and others have shown that the degree of compensation by collateral vessels, even when they have matured after a period of rapid growth, reaches only about 40% of the maximal conductance of the artery they had replaced.[1,2] Fluid shear stress initiates the activation of endothelial cells and modulates processes, which control attraction of circulating cells to the collateral wall.[3] Monocytes were shown to play a pivotal role during arteriogenesis.[4,5]

Further elucidation of the molecular pathways leading to a functional “natural bypass” could be the basis for stimulating the nonoptimal processes to produce full restoration of arterial function.

The undisturbed arteriogenic process only leads to an incomplete restoration of function, probably because of the early restoration of fluid shear stress (FSS), which is related to the cube root of the expanding radius. This early growth-induced decline of the FSS level was prevented in a previous experiment by creating an arterio-venous (AV)-shunt to drain most of the collateral flow into the venous system[3,6] thereby reestablishing a method invented by Holman in 1949.[7] As a consequence, a long-lasting growth of collateral vessels completely restored (and overshot) physiological function of the occluded artery. However, the Rho kinase inhibitor Fasudil abolished the shunt effect completely.[6]

In a genome-wide screening of mRNA abundance in growing collaterals of rats we identified *actin-binding Rho activating protein* (*Abra*). Abra (also known as Stars) is a muscle specific actin-binding protein capable of stimulating *SRF*-dependent transcription through a mechanism involving RhoA and actin polymerization.[8] In cardiac tissue *Abra* mRNA is upregulated in response to pressure overload.[8,9] Recently it was shown that Abra is involved in human

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skeletal muscle hypertrophy and atrophy.[10] Forced overexpression of Abra in mouse heart tissue results in an increased sensitivity to biomechanical stress stimuli leading to cardiac hypertrophy.[11] However, its role in blood vessels has not been determined. In light of the requirement of Rho signaling during arteriogenesis, we hypothesized that a fluid shear stress–induced upregulation of *Abra* initiates collateral remodeling. To test this hypothesis, the upregulation in growing collaterals was confirmed by independent techniques like qRT-PCR and Western Blot. With the objective to explore the cellular localization of Abra in the vascular wall we performed in situ hybridization and immunohistochemistry. The stimulating effect of Abra on cultured vascular cells was examined in a proliferation assay. Subsequently, we quantified the arteriogenic response in vivo after a local adenoviral overexpression in rabbits as well as a targeted deletion of *Abra* in mice.

Our findings suggest an important role of Abra during the initiation of arteriogenesis. The previously described features of Abra could in part be extended to arteriogenesis. Finally we propose a position for the novel arteriogenesis gene in the existing arteriogenesis pathways and thereby we contribute to a more comprehensive understanding of the process.

Methods

An extended methods section is available in the supplemental materials (available online at http://atvb.ahajournals.org).

Animal Models

The study was performed according to Section 8 of the *German Law for the Protection of Animals*, which confirms to the U.S. National Institutes of Health (NIH) guidelines.

New Zealand White rabbits (Charles Riber, Kissleg, Gergmany) were used for hemodynamic analyses. Spraque Dawley rats (Harlan Winkelmann, Borchen, Germany) served as a model for the genome-wide expression studies, and $Abra^{-/-}$ mice (a kind gift from Dr E. Olsen, University of Texas Southwestern Medical Center, Dallas, Texas) were used to study the arteriogenic effect of targeted deletion of the *Abra* gene.

Cell Culture, Proliferation Assay

Porcine aortic endothelial cells (PAECs) were obtained from the aorta as previously described.[12] To investigate proliferative activity the MTT Cell Proliferation Assay (ATCC) was used.

RNA Isolation, Quantitative Real-Time PCR, and Microarray Analysis

Seven days after surgery total RNA was isolated (RNeasy Mini kit, Qiagen) from dissected collaterals or other organs. qRT-PCR was performed, and the relative amount of target mRNA normalized to 18S RNA was calculated as previously described.[13]

The data discussed in this publication have been deposited inNCBIs Gene Expression Omnibus[14] and are accessible through GEO Series accession number GSE16359. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16359).

Adenoviral Production

Recombinant adenoviruses expressing Abra were generated using the ViraPower Adenoviral Expression system (Invitrogen). Adenoviral plasmids were transfected into 293A cells using Metafectene (Biontex). Adenovirus was purified from the cells using the Vivaspin kit (Vivascience). Titer-estimation was performed using QuickTiter Adenovirus Titer Immunoassay Kit (Cell Biolabs Inc).

In Situ Hybridization

DIG-labeled RNA probes corresponding to the sense and antisense strand of the *Abra* cDNA were prepared by in vitro transcription using DIG RNA Labeling Kit SP6/ T7 (Roche) according to the manufacturers' protocol. In situ hybridization was performed on paraffin sections of the rat M. quadriceps as described.[15] Detection was done with anti-DIG antibody (Roche) and visualized by NBT/BCIP staining (Roche). Sections were counterstained with eosin.

Western Blot Analysis

Western blotting was performed on protein extracts of collateral vessels or cultured cells with specific antibodies as described previously.[16] Used antibodies and concentrations: Abra 1:500 (Davids Biotechnologie, from chicken for PAECs and tissue, from rabbit for PSMCs), Vinculin 1:800 (Sigma).

Immunohistochemistry

Immunostaining was performed as previously described.[17] Sections were viewed with a confocal microscope (Leica TCS SP).

Statistical Analysis

All values are expressed as mean±SEM. Two treatment groups were compared by the unpaired Student *t* test. One-way ANOVA (Prism, GraphPad Software Inc) was performed for maximum collateral conductances. Probability values less than 0.05 were considered as statistically significant.

Results

Actin-Binding Rho-Activating Protein (*Abra*) Is Upregulated in FSS-Stimulated Collateral Arteries

To analyze the gene expression pattern of FSS-induced collateral growth we adapted the arterio-venous (AV)-shunt model to rats. Animals were subjected to femoral artery ligature (FAL) in both legs and a one-sided additional AV-shunt for increased FSS in the collateral arteries. In search for genes that may be involved in the mediation of FSS-induced arteriogenesis, we performed gene expression profiling of growing collaterals in rats using microarray analysis.

A large set of reproducibly differential genes was detected at high statistical significance between sham-treated and shunt-treated collateral arteries. A heat map shows the reproducibility of these findings between the different individual arteries that constitute the 2 different groups. Hierarchical clustering of the top 100 of the most differential genes between sham and shunt treated collateral arteries is given in supplemental Figure I. A subset of 354 transcripts demonstrating statistically significant differences (log2 ratio >4.0 or <-4.0, $P<0.05$) in abundance between AV-shunt and sham collaterals was used for pathway analysis. Gene Set Enrichment Analysis (GSEA)[18] and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) showed an enrichment of transcripts displaying a role in remodeling of the actin cytoskeleton, an activation of the Rho/ROCK-pathway and the calcium signaling pathway (supplemental Table II).

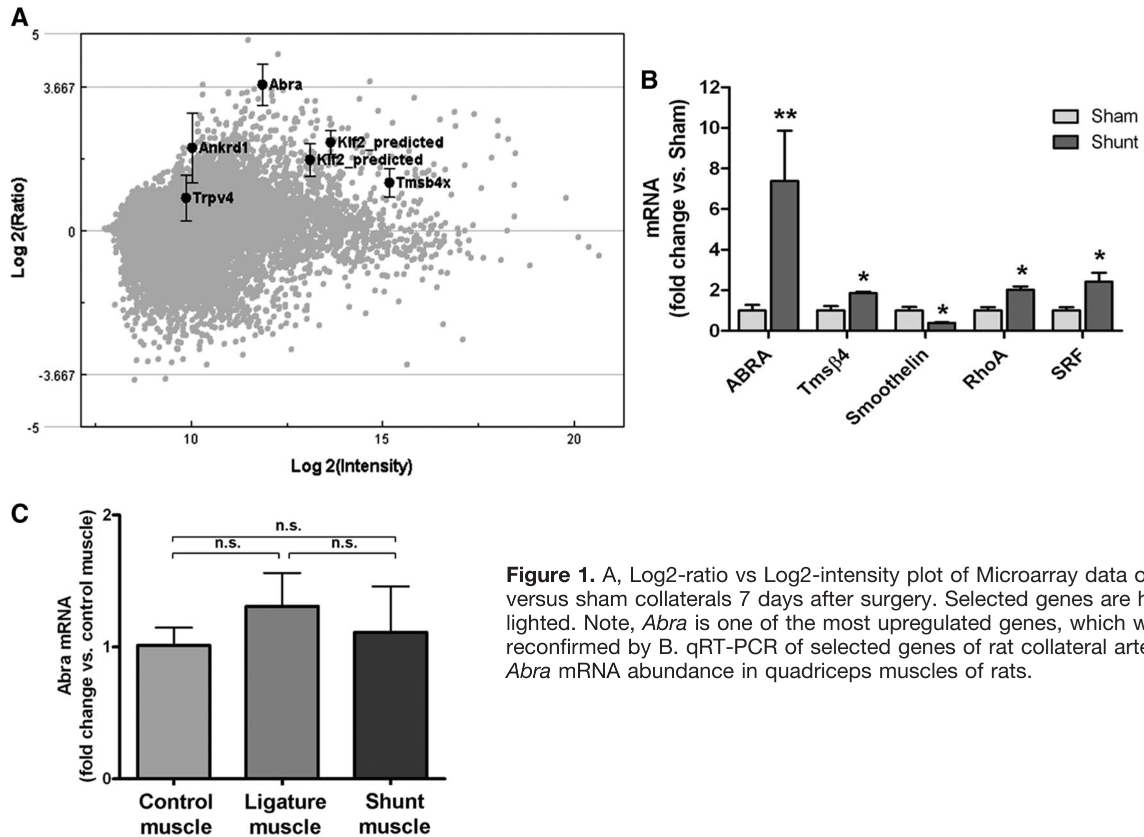

Figure 1. A, Log2-ratio vs Log2-intensity plot of Microarray data of shunt versus sham collaterals 7 days after surgery. Selected genes are highlighted. Note, *Abra* is one of the most upregulated genes, which was reconfirmed by B. qRT-PCR of selected genes of rat collateral arteries C, *Abra* mRNA abundance in quadriceps muscles of rats.

One of the most upregulated genes was *Abra* (Figure 1A). qRT-PCR confirmed upregulation of *Abra, RhoA, serum response factor* (*SRF*), and *Tmsb4* in growing collateral vessels. Smoothelin, a marker for SMC differentiation, was significantly downregulated in shunt collaterals (Figure 1B).

The amount of *Abra* transcripts in the surrounding skeletal muscle was unchanged in control-, ligature-, or shunt-treated animals (Figure 1C). Furthermore, we tested rat aortic smooth muscle cells (RASMCs), which were exposed to cyclic stretch for 0.5 to 24 hours, for *Abra* mRNA abundance and did not find any difference when compared to untreated controls (supplemental Figure II).

Abra Is Expressed in Blood Vessels

In contrast to the previously identified restriction of endogenous Abra to cardiac and skeletal muscle,[8] we demonstrated that *Abra* is also transcribed in blood vessels using gene-specific primers for end point PCR. To exclude striated muscle contamination we investigated also those arteries which are not surrounded by skeletal muscle. *Abra* transcripts were detected in the heart, quadriceps muscle, and in all examined arteries such as the aorta, iliac arteries, and small collateral vessels (Figure 2A). The cellular localization in the vessel wall was studied by in situ hybridization with a DIG-labeled *Abra* RNA antisense probe on paraffin sections of quadriceps muscles of rats. mRNA transcripts were detected in the collateral vessels 7 days after AV-shunt treatment in ECs as well as in SMCs (Figure 2B). We immunostained cryosections of collateral arteries of the quadriceps muscle to correlate the new transcriptional localization with the corresponding protein expression. The specificity of the antibody was confirmed by the lack of signal when Abra peptide, used for the generation of the antiserum, was added before staining. In the Western Blot, a specific signal appeared in COS1 cells transfected with *Abra* carrying plasmid. Previous observations of a partial overlap of the Z-line in longitudinal sections of skeletal muscle could be confirmed by coimmunostaining with anti-Abra and anti α-actinin (supplemental Figure III).

In the vasculature, Abra protein was mainly present in the cytosol of SMCs and in the nuclei of ECs. (Figure 2C). In Western Blot as well as when comparing cross-sections of collaterals of shunt treated or FAL treated rats, highest Abra expression was also found after shunt treatment.

To examine the transmission of FSS exposed to the endothelium to Abra expression in the media, shunt treated rats received L-NAME (30 mg/kg BW/d) in the drinking water. The blockage of all isoforms of NO synthases abolished the stimulatory effect of FSS on collateral growth (Figure 2E) and resulted in completely repressed *Abra* transcription (Figure 2F) providing a potential transduction mechanism of FSS.

Abra Overexpression Induces Proliferation Activity of SMCs via Rho/ROCK

A crucial process during arteriogenesis is increased proliferation mainly of SMCs but also of ECs. Therefore we investigated whether *Abra* overexpression triggers prolifera-

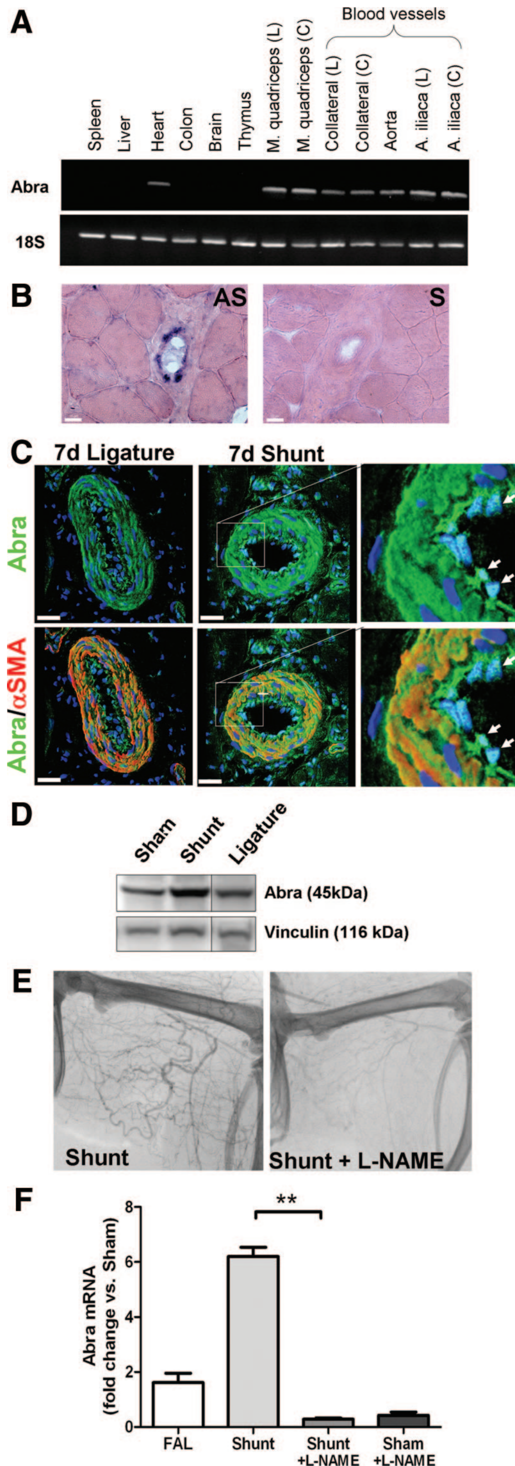

Figure 2. A, *Abra* mRNA detection by end point PCR in rat tissue (L indicates ligature; C, untreated control). B, Cellular localization of *Abra* transcripts by in situ hybridization of shunt treated collateral vessels in rats. (AS indicates antisense probe; S, sense probe. Scale bars=20 μm) C, Cross sections of rat collateral vessels 7 days after indicated treatment (Abra, green; αSMA, red; scale bars=30 μm). Arrows in magnifications of micrographs depict nuclear localization of Abra in endothelial cells. D, Western blot analysis of *Abra* protein abundance in rat collaterals 7 days after surgery. Membranes were reprobed for Vinculin to ensure equal loading. E, Representative angiographies of rat hindlimbs 7 days after indicated treatment. F, Effect of indicated treatment on *Abra* transcription in rat collateral arteries.

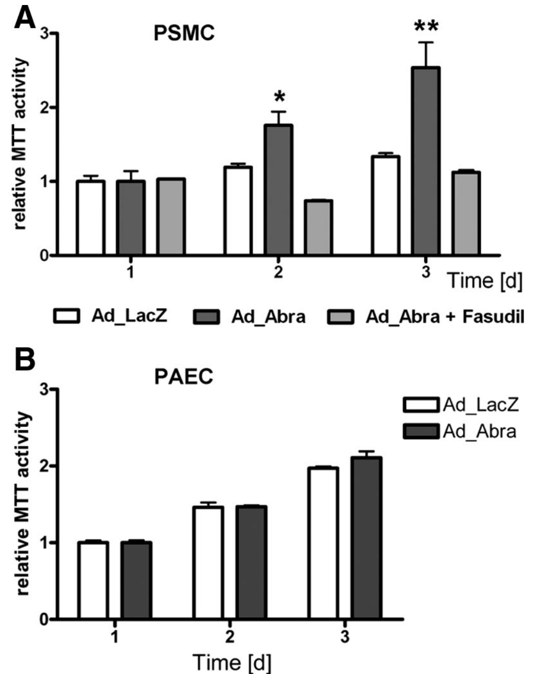

Figure 3. A, Proliferation activity of *Abra*-transduced porcine SMCs (PSMCs) showed a significant increase of MTT activity after 3 days (>90%) which could be abolished by Fasudil. B, *Abra*-transduced ECs did not show increased proliferation activity compared to Ad_LacZ controls (values are given in mean±SEM, n=8; *$P<0.05$; **$P<0.01$ vs Ad_LacZ).

tive and metabolic activity of SMCs and ECs using MTT cell proliferation assay.

Abra overexpressing porcine SMCs showed a 47.5% increase of relative proliferative activity on day 2 ($P<0.05$) compared to Ad_LacZ–transduced controls (n=8). On day 3 the proliferative activity was almost doubled (90%, $P<0.01$). Rho kinase inhibitor Fasudil inhibited Abra-induced proliferative activity, indicating an involvement of Rho signaling in the process (Figure 3A). In contrast, porcine aortic endothelial cells (PAECs) in culture did not show significant changes in proliferation activity after *Abra* transduction compared to Ad_LacZ controls on day 2 and 3 (Figure 3B). In both experiments, immunostaining and Western Blot confirmed recombinant Abra expression (supplemental Figure IV). Proliferative activity in *Abra*-transduced SMCs was confirmed in an A10 rat cell line (supplemental Figure V).

Abra Modulation In Vivo by a Local Intracollateral Adenoviral Gene Transfer

To address the functional implications of Abra on arteriogenesis in vivo we modulated its expression by adenoviral gene transfer. A recombinant *Abra* adenovirus under the control of a CMV promoter (Ad_*Abra*) was locally injected into the collaterals of rabbits. These experiments were done after an initial FAL without AV-shunt treatment to investigate whether gene transfer of *Abra* alone can substitute for the effect of FSS.

Virus solution was injected into the collateral system of the rabbit hindlimb. A temporary occlusion proximal of the ligature interrupted the blood flow and allowed the virus to incubate for 30 minutes inside the collateral arteries before it

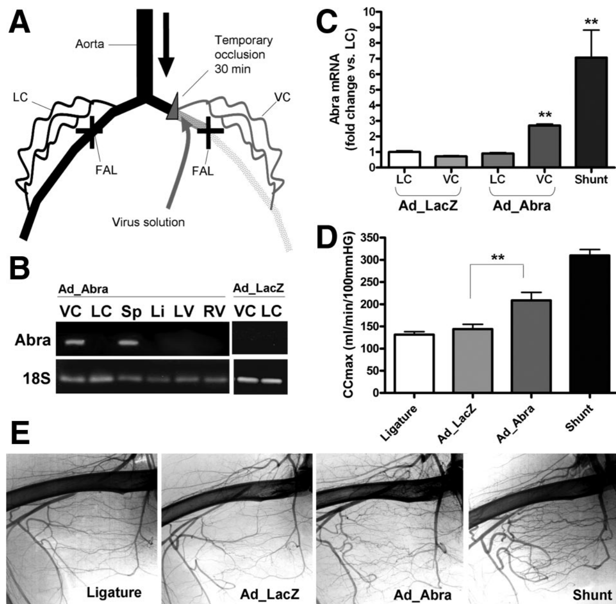

Figure 4. A, Scheme of local adenoviral gene transfer in the collateral circulation of the rabbit hindlimb. B, Transgene specific RT-PCR of RNA from either Ad_*Abra*– or Ad_LacZ–transduced rabbit organs (collaterals of the virus side [VC], collaterals of the ligated side [LC], spleen [Sp], Liver [Li], left ventricle [LV], right ventricle [RV]). C, qRT-PCR showing *Abra* mRNA abundance of isolated collaterals of either Ad_*Abra*– or Ad_LacZ– (4×10^{10} ifu) treated rabbits in comparison to shunt collaterals (n=3). D, Maximum collateral conductance (CC_{max}) in rabbit hindlimbs after indicated treatments (Values are given in mean±SEM, n=6, ***P*<0.01). E, Representative angiographies of rabbit hindlimbs 7 days after indicated treatment.

was opened again (Figure 4A). A transgene specific RT-PCR confirmed a local restriction to the site of transduction. Viral transcripts were detected in the collaterals of the virus side (VC) but not in those tissues of the control ligated side (LC). An accumulation of excessive virus was observed in the spleen. No *Abra* viral transcripts appeared in collaterals of Ad_LacZ treated rabbits (Figure 4B).

A maximum of 2.5-fold forced local overexpression of *Abra* could be reached by 4×10^{10} ifu of Ad_*Abra* (Figure 4C; n=3). This resulted in a significant improvement of maximum collateral conductance to 230.1±18.6 mL/min/100 mm Hg (versus Ad_LacZ: 144±10.6 mL/min/100 mm Hg) 7 days after gene transfer, which represents a partial substitution of the effect of AV-Shunt treatment (306±15 mL/min/100 mm Hg; Figure 4D). Corresponding angiographies of virus-treated rabbit hindlimbs illustrate the collateral growth caused by *Abra* overexpression (Figure 4E).

Arteriogenesis Is Impaired in Mice Lacking *Abra*

To test the "loss of function" we investigated the arteriogenic response of $Abra^{-/-}$ mice. Targeted deletion was confirmed by the lack of *Abra* mRNA in homozygous mutant skeletal muscles by RT-PCR (Figure 5A). Quantification of tissue perfusion in the distal hindlimbs of femoral artery ligated $Abra^{-/-}$ mice or nontransgenic littermates (wild-type, WT) by Laser Doppler blood flow demonstrated an impaired arteriogenesis in $Abra^{-/-}$ mice.

In both, $Abra^{-/-}$ and WT mice (n=15) with the CL57BL/6 background, right to left ratio decreased immediately after occlusion (1.01±0.02 to 0.08±0.009 [$Abra^{-/-}$] versus 1.01±0.017 to 0.08±0.009 [WT]). A continuous blood flow recovery to up to 91% was observed during the whole observation period of 3 weeks in control animals, whereas $Abra^{-/-}$ did not further improve 59% of blood flow recovery after 14 days (Figure 5B and 5C).

To define whether Abra contributes to angiogenesis or collateral growth in our model we studied collateral anatomy in the complete adductor muscle of WT or *Abra* mice and performed morphometry: Diameter (46.3±3.4 μm versus 48.8±2.8 μm), wall area (1076±154 μm^2 versus 1123±167 μm^2), and wall area of preexisting arterioles did not differ between WT and $Abra^{-/-}$ mice. Collateral diameter was increased after 21 days of ligature in both groups, but in $Abra^{-/-}$ mice the diameter was significantly smaller than in WT ($Abra^{-/-}$: 65.2±2.2 μm; WT: 77.0±3.5 μm). Even more notable, 21 days after ligature, collateral wall area did not increase in $Abra^{-/-}$ mice at all but significantly differed from WT (1397±129 versus 1861±182 μm^2, $P<0.01$; Figure 5D). These findings confirmed the above perfusion data and indicate an incomplete regeneration of collateral arteries in *Abra*-deficient mice.

Capillary density in the calf muscle was also assessed and provided additional evidence for the arteriogenesis-specificity of our hindlimb model. In calf muscles of unligated WT mice capillary density was 1.67±0.14 (in capillary/fiber) and 1.74±0.12 in unligated $Abra^{-/-}$. 21 days after ligation the capillary/fiber ratio in calf muscles showed a slight but insignificant increase in both groups (WT: 1.78±0.05; KO: 1.82±0.12) suggesting the absence of an angiogenic stimulus in these mice (Figure 5D).

Abra-Induced Rho Signaling and Actin Dynamics in FSS-Induced Arteriogenesis

To correlate *Abra* overexpression with changes in RhoA expression, we probed for RhoA expression in FSS-stimulated and *Abra*-overexpressing collateral vessels of rats as well as in *Abra*-deficient mice. Representative micrographs illustrate increased RhoA expression after shunt surgery, which can also be induced by forced adenoviral *Abra* overexpression (Figure 6A). Contrary to that, RhoA is decreased in *Abra*-deficient mice (Figure 6B).

To determine actin polymerization in FSS-induced arteriogenesis we performed a specific staining for G-actin and F-actin in shunt-treated collateral arteries. As expected, the dedifferentiated state of SMCs is characterized by a decrease in F-actin and an increase in G-actin (Figure 6C).

Discussion

We have identified Abra as a novel initial regulator of arteriogenesis. In a rat model of chronically elevated fluid shear stress (FSS) *Abra* is upregulated in growing collaterals. The results of our present study show that a local intracollateral adenoviral overexpression of *Abra* improves collateral conductance after FAL in rabbits. Targeted deletion of *Abra* in mice leads to impaired arteriogenesis.

It is known that among the physical forces that control the size of the arterial tree, FSS is the most important one.[19–23] We have recently developed a new small animal model (reminiscent of a large-animal experiment reported already in

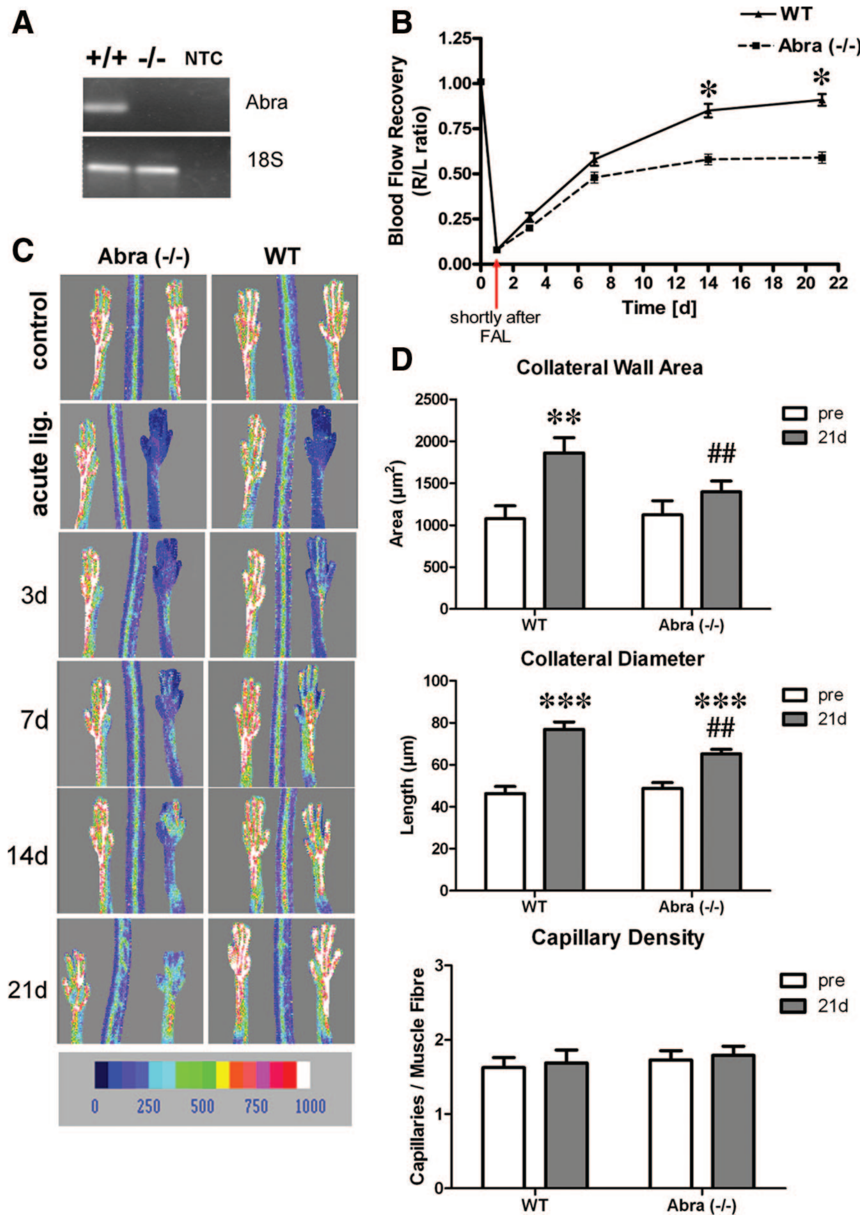

Figure 5. A, End point RT-PCR from RNA of skeletal muscle showing no *Abra* transcripts in $Abra^{(-/-)}$ mice compared to nontransgenic littermates (+/+). NTC indicates no template control. B, Impaired blood flow recovery in right femoral artery ligated $Abra^{-/-}$ mice, expressed as Laser Doppler perfusion right to left ratio. (Values are given in mean±SEM; **P*<0.05 vs WT) C, Representative color-coded laser Doppler perfusion images. D, Morphometric measurements of collateral arteries and capillaries in gene-targeted mice (n ≥15; values are given in mean±SEM; ##*P*<0.01 vs WT, ****P*<0.001, ***P*<0.01, **P*<0.05 vs preexistent).

1949 by Holman[7]), where FSS was maximized by creating an arterio-venous shunt between the distal stump of the occluded femoral artery and the accompanying vein.[3,24] In the present study we tried to elucidate the regulators/mediators of collateral growth initiated by high fluid shear stress. In a comparative study we analyzed gene expression pattern of collateral arteries of rats subjected to shunt treatment and preexistent collateral arteries.

We selected differentially expressed Abra, a muscle-specific actin-binding protein capable of stimulating *SRF*-dependent transcription through a mechanism involving RhoA and actin polymerization,[8] which we found to be 7-fold overexpressed (qRT-PCR) in growing collateral arteries. This is interesting in the light of previous findings that Abra is stress-inducible and markedly upregulated during hypertrophic growth of the heart in response to calcineurin activation as well as pressure overload.[8,9] Its subcellular localization in the Z-line of the sarcomere suggests a stress-dependent control of expression,[8] and therefore we hypothesized a potential involvement of Abra during the FSS-induced stimulation of arteriogenesis. In the present study we could assign a novel localization and a new function to Abra, thereby enlarging the previously described restriction to striated muscle. Under conditions of high shear stress *Abra* is strongly upregulated, both on the transcriptional as well as on the translational level in the cytoplasm of smooth muscle cells of growing collaterals. A minor degree of FSS induced overexpression is also found in the endothelium. Our claim that *Abra* is induced by FSS is supported by the observation that rat aortic smooth muscle cells (RASMCs), which were exposed to cyclic stretch for 0.5 to 24 hours, do not respond with altered *Abra* mRNA abundance.

To investigate the functional role of Abra during arteriogenesis we modulated its expression. Here we show that *Abra* overexpression stimulates SMC proliferation in culture. Abra may act as a direct SMC mitogen or it contributes indirectly

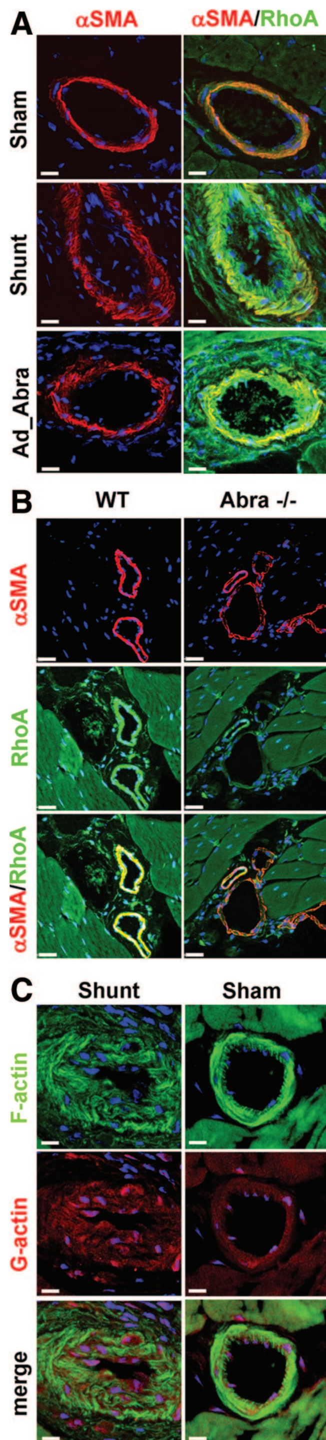

Figure 6. Immunofluorescence confocal microscopy of collateral vessels in rats (A and C) and mice (B) was performed 7 days after the indicated treatments, using antibodies (A and B) against RhoA (green) and the SMC marker αSMA (red), (C) against F-actin (green) and G-actin (green). Nuclei were counterstained with Draq5 (blue; scale bars=40 μm).

to a reprogramming of a distinct growth phenotype characterized by the ability to replicate in an autonomous mitogen-independent manner as it was shown for embryonic aortic SMCs in culture.[25,26]

Adenoviral overexpression of *Abra* in vivo in rabbits with acute femoral artery occlusion resulted in a significant 60% increase of maximum collateral conductance 7 days after gene transfer when compared to LacZ control, which represents a partial substitution of the effect of AV-Shunt treatment. The maximum dose (4×10^{10} ifu) of virus, which could be administered in the lumen of the collateral bed, led to a 2.5-fold increase of collateral *Abra* expression when compared to sham-treated collateral arteries. This fractional amount of the FSS provoked *Abra* expression (up to 7-fold) in the shunt model improved the natural response but did not completely achieve the full compensation of the occluded femoral artery. In contrast, quantification of tissue perfusion in the distal hindlimbs of $Abra^{-/-}$ mice with femoral artery occlusion or nontransgenic littermates by Laser Doppler blood flow demonstrated an impaired arteriogenesis in $Abra^{-/-}$ mice. This was further confirmed by morphometry. Normal capillary density in the distal hindlimb in both groups excludes an angiogenic stimulus.

Because we found *Abra* strongly upregulated under conditions of artificially high FSS we are currently establishing the shunt surgery in gene-targeted mice.

The well-established abilities of Abra to activate Rho signaling, to influence actin dynamics as well as cytoskeletal integrity, and to initiate SRF-dependent gene transcription[8] provide potential mechanisms to account for the improvement of collateral growth.

We know from previous studies that the Rho-pathway is involved in flow-related remodeling of small arteries[27] and in particular in arteriogenesis,[6] because the Rho kinase blocker Fasudil abolished the beneficial effect of shunt treatment in rabbits. It is therefore suggestive that the Abra should play an important role in arteriogenesis. Increased RhoA expression in FSS-stimulated collaterals as well as in Ad_*Abra*–treated collaterals support a causal connection of Abra and RhoA in arteriogenesis.

In addition, changes in actin dynamics are hallmarks for arteriogenesis: The phenotypic transition in smooth muscle cells during arteriogenesis from the contractile to the synthetic and proliferative state is characterized by the lack of actin filaments, which is a result of downregulation of actin transcription as well as the degree of actin polymerization. In our experiments of chronically elevated FSS we prolonged the dedifferentiated state of smooth muscle cells that is characterized by differential expression of the actin-(de)polymerizing proteins (destrin, cofilin1, cofilin2, and transgelin2[3]). A specific staining of F-actin and G-actin supports this observation: F-actin is more fragmented, whereas G-actin accumulates in shunt vessels compared to sham-treated collaterals.

However, Abra promotes the formation of F-actin, and Rho activation leads to actin polymerization. This apparent contradiction corresponds with and extends previous findings derived from forced *Abra* overexpression in the heart. Increased expression of *Abra* in response to stress stimuli—FSS in our model—may initially serve as a compensatory response to increased actin content to maintain cytoskeletal integrity and sustain arterial function.[11] But excessive expression leads to adaptation, which results in adverse cardiac remodeling in mouse models of cardiac hypertrophy[11] or, as we could demonstrate in rat and rabbit models of FAL, to

beneficial collateral growth. Interestingly, *Abra* overexpression in both models is accompanied by activation of SRF-dependent fetal cardiac genes[11] (and our results from microarrays, supplemental Table II).

Abra initiates SRF-dependent transcription. Apart from previous findings of SRF-dependent transcription during arteriogenesis (reviewed in[28]), we were able to demonstrate that SRF itself is upregulated under conditions of high FSS. RhoA-dependent regulation of the actin cytoskeleton selectively regulates SMC differentiation marker gene expression by modulating SRF dependent transcription.[29]

The question how the signals, generated in endothelial cells, are translated into a growth stimulus for the smooth muscle layer of the media that is separated from the endothelium by the internal elastic lamina, remains unsolved. None of the already known endothelial stress–responsive proteins (Klf2,[30] PECAM-cadherin-VEGFR2 complex,[31] Trpv4[24]) explain the proliferation of the SMCs of the media. We as well as others were unable to find endothelial-to-smooth muscle junctions in collaterals,[32,33] which reduce the means of communication to diffusible transmitters, like NO, oxygen radicals, endothelin, and diffusible factors like VEGF. However, because monocytes play an important role in arteriogenesis and their blockade leads to impaired arteriogenesis, NO from iNOS may be a candidate. The blockage of all sources of NO by L-NAME completely abolished the beneficial shunt effect[6] and resulted in significantly decreased *Abra* transcription. These findings suggest that NO is involved in the FSS mediation to the media and results in *Abra* induction, which provides a potential explanation of the unexpected mitogenic effect of NO.[34,35]

One of the earliest events in arteriogenesis is the FSS-dependent activation of mechanosensitive *transient receptor potential cation channel, subfamily V, member 4* (*Trpv4*),[24] a Ca^{2+} permeable ion channel. To further elucidate the upstream signaling we analyzed *Abra* promoters of 7 different species including chicken and opossum in silico with genomatix software to find transcription factor (TF) binding sites. Two closely spaced potential regulatory units for ATF4 and MEF2 upstream of transcription start sites were detected (supplemental Figure VI). The involvement of MEFs in many muscle specific regulatory circuits[36,37] and MEF2-dependent *Abra* transcription has recently been shown.[11] In addition we identified a DRE-site in the rat *Abra* promoter that binds Kv channel interacting protein 3 (Kcnip3, also known as Dream). The fact that binding sites of 2 of the 3 major calcium dependent transcriptional effectors (NFAT, MEF2, DREAM)[38] are present within the *Abra* promoter point to a potential Ca^{2+}-dependent regulation. In a recently published microarray analysis of gene expression in mouse aorta an involvement of the calcium signaling pathway was demonstrated for the process of atherosclerosis. In this context Ca^{2+} seems to be involved in the monocyte chemoattractant protein-1 production,[39] which is a prerequisite for arteriogenesis as well.[5,40]

Taken together, our data suggest a functional implication of Abra for arteriogenesis. We showed an FSS-dependent upregulation of *Abra* in collateral vessels and a functional consequence for collateral growth. The previously described features of Abra were, in most instances, extended to arteriogenesis. Finally we propose a position for the novel arteriogenesis gene in the existing arteriogenesis pathways as being a link between the mechanical stimulus of FSS and the proliferation of SMCs, thus contributing to a more comprehensive understanding of the process.

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Disclosures

None.

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