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


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 ligation (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 blockade 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 system3,6 thereby reestablishing a method invented by Holman in 1949.7 As a consequence, a long-lasting growth of collateral vessels completely restored (and overshoot) 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 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
skeletal muscle hypertrophy and atrophy.\textsuperscript{10} Forced overexpression of Abra in mouse heart tissue results in an increased sensitivity to biomechanical stress stimuli leading to cardiac hypertrophy.\textsuperscript{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 adventitial overexpression in rabbits as well as a targeted deletion of \textit{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.

\section*{Methods}

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

\subsection*{Animal Models}

The study was performed according to Section 8 of the \textit{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 to study the arteriogenic effect of targeted deletion of \textit{Abra} in mice.

\subsection*{Cell Culture, Proliferation Assay}

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

\subsection*{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.\textsuperscript{13} The data discussed in this publication have been deposited in NCBI Gene Expression Omnibus\textsuperscript{14} and are accessible through GEO Series accession number GSE16359. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16359).

\subsection*{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).

\subsection*{In Situ Hybridization}

DIG-labeled RNA probes corresponding to the sense and antisense strand of the \textit{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 M4. quadriceps as described.\textsuperscript{15} Detection was done with anti-DIG antibody (Roche) and visualized by NBT/BCIP staining (Roche). Sections were counterstained with eosin.

\subsection*{Western Blot Analysis}

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

\subsection*{Immunohistochemistry}

Immunostaining was performed as previously described.\textsuperscript{17} Sections were viewed with a confocal microscope (Leica TCS SP).

\subsection*{Statistical Analysis}

All values are expressed as mean±SEM. Two treatment groups were compared by the unpaired Student \textit{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.

\section*{Results}

\subsection*{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 ligation (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)\textsuperscript{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).
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, 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-
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...
occlusion (1.01±0.02 to 0.08±0.009 [Abra<sup>-/-</sup>] 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<sup>-/-</sup> 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<sup>2</sup> versus 1123±167 μm<sup>2</sup>), and wall area of preexisting arterioles did not differ between WT and Abra<sup>-/-</sup> mice. Collateral diameter was increased after 21 days of ligation in both groups, but in Abra<sup>-/-</sup> mice the diameter was significantly smaller than in WT (Abra<sup>-/-</sup>: 65.2±2.2 μm; WT: 77.0±3.5 μm). Even more notable, 21 days after ligation, collateral wall area did not increase in Abra<sup>-/-</sup> mice at all but significantly differed from WT (1397±129 versus 1861±182 μm<sup>2</sup>, 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<sup>-/-</sup> mice. 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
1949 by Holman), where FSS was maximized by creating an arterio-venous shunt between the distal stump of the occluded femoral artery and the accompanying vein. 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, 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. Its subcellular localization in the Z-line of the sarcomere suggests a stress-dependent control of expression, 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
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.\textsuperscript{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\textsuperscript{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\textsuperscript{−/−} mice with femoral artery occlusion or nontransgenic littermates by Laser Doppler blood flow demonstrated an impaired arteriogenesis in Abra\textsuperscript{−/−} 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\textsuperscript{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\textsuperscript{27} and in particular in arteriogenesis,\textsuperscript{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\textsubscript{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, coflin1, coflin2, and transgelin2\textsuperscript{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.\textsuperscript{11} But excessive expression leads to adaptation, which results in adverse cardiac remodeling in mouse models of cardiac hypertrophy\textsuperscript{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\(^\text{11}\) (and our results from microarrays, supplemental Table II).

Abra initiates SRF-dependent transcription. Apart from previous findings of SRF-dependent transcription during arteriogenesis (reviewed\(^\text{in}\)\(^\text{29}\)), 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.\(^\text{29}\)

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,\(^\text{30}\) PECAM-cadherin-VEGFR2 complex,\(^\text{31}\) Trpv4\(^\text{28}\)) 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,\(^\text{32,33}\) 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\(^\text{6}\) 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.\(^\text{34,35}\)

One of the earliest events in arteriogenesis is the FSS-dependent activation of mechanosensitive transient receptor potential cation channel, subfamily V, member 4 (Trpv4)\(^\text{24}\) 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\(^\text{36,37}\) and MEF2-dependent Abra transcription has recently been shown.\(^\text{11}\) In addition we identified a DRE-site in the rat Abra promoter that binds Kcv 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)\(^\text{38}\) 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,\(^\text{39}\) which is a prerequisite for arteriogenesis as well.\(^\text{5,40}\)

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.

References


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In the article, “Actin-Binding Rho Activating Protein (Abra) Is Essential for Fluid Shear Stress-Induced Arteriogenesis” by Troidl et al, which appeared in the December 2009 issue of the journal (Arterioscler Thromb Vasc Biol. 2010;29:2093–2101; DOI: 10.1161/ATVBAHA.109.195305), the publisher omitted several important errors from the final, printed version of the article:

1. Page 2093, affiliation, the current affiliation for I.P. should have included UCL Institute of Child Health, to read: “Molecular Haematology and Cancer Biology, UCL Institute of Child Health, London, UK;” the current affiliation for W.S. should have included the Division of Vascular and Endovascular Surgery, University of Regensburg, to read: “the Division of Vascular and Endovascular Surgery, University of Regensburg, Germany”

2. Page 2094, Methods, Animal Models, 1st line of the 2nd paragraph, Charles Riber, Kisslegg, Germany should have been noted as the supplier of New Zealand White Rabbits, and Harlan Winkelmann, Borchen, Germany should have been noticed as the supplier of Spraque Dawley rats.

3. Page 2097, 2nd column, 2nd paragraph, lines 5-6, “wall thickness” should have been removed

4. Page 2100, Sources of Funding, the Messer Foundation should have been inserted

5. Page 2101, reference 32, Academic should have appeared as Academic

The online version has been corrected.

The publisher sincerely regrets the errors.

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Supplement Material

Actin-binding Rho activating protein (*Abra*) is essential for fluid shear stress-induced arteriogenesis

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This pdf file includes

Extended material and methods section

Figure Legends

References

Tables T1 and T2

Figures S1 to S6
Extended Material and Methods section

Animal models

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

24 male New Zealand White rabbits (Charles River; 3.0 +/-0.3 kg body weight) were randomly assigned to one of four groups. Both Femoral arteries were ligated in every group, the following further treatments were added: one-sided AV-Shunt-treatment as described earlier\textsuperscript{1-3}, one-sided intra-collateral infusion of virus-solution (4x10\textsuperscript{10} ifu Ad_Abra or Ad_LacZ) or control ligature. The surgical procedures were carried out under anesthesia with ketamine hydrochloride (40 mg/kg body weight for rabbits and 100 mg/kg body weight for rats) and xylazine (4 mg/kg for rabbits and 3 mg/kg for rats) administrated i.m.. To prevent pain buprenorphine (50 µg/kg for rabbits and 20 µg/kg for rats) was used.

Maximum collateral conductance was assessed seven days after treatment and for post mortem angiographies gelatine based barium sulphate contrast medium was infused as described \textsuperscript{2 4}. Collateral arteries were dissected from the surrounding muscle for RNA isolation.

Arterio-venous shunt experiments, post mortem angiographies and isolation of collateral vessels were carried out in male Spraque-Dawley rats (250-300 g, Charles River Laboratories, Sulzfeld, Germany) as described for rabbits. For NO blockage, a subgroup of the shunt treated rats received L-NAME in the drinking water (30mg/kg body weight/day). Quadriceps muscles were collected for immunohistochemistry or \textit{in situ} hybridisation.

Mice: To study the arteriogenic effect of targeted deletion of Abra C57Bl/6 mice For morphometric analysis of the collaterals, serial cryosections (6 µm) of adductor muscles were stained with FITC-conjugated monoclonal antibody against α-smooth muscle (α-SM) actin (Sigma-Aldrich) and nuclei were stained using DAPI (Mobitec). Collaterals of both ligated and non-ligated femoral arteries were identified and the diameters and wall areas were
determined using the ImageJ 1.38p software (http://rsb.info.nih.gov/ij/). Capillary density in the Mm. gastrocn. was determined after CD31 staining.

**Cell culture, Proliferation Assay, Cyclic stretch**

Porcine aortic endothelial cells (PAEC) were obtained from the aorta as previously described and cultured in DMEM, 10% FCS, 20 mM L-Glutamine, 10 mM sodium-pyruvat, 10 mM non-essential amino acids, and 10 mM penicillin/streptomycin. Porcine smooth muscle cells (PSMC) were isolated from the medial layer of the thoracic aorta and grown in DMEM, 10% FCS, and 10 mM penicillin/streptomycin. To investigate proliferative activity the MTT Cell Proliferation Assay (ATCC®) was used. Cells were seeded in 6-well cell culture dishes (Ø 3.2 cm) and transduced with 1x10^5 ifu/well of recombinant adenovirus Ad_Abra (n=8) or Ad_LacZ (n=4). In order to inhibit Rho-kinase 10 µM Fasudil was added to the media of Ad_Abra transduced PSMC. Prior to proliferation tests, cells were serum-starved for 24 hours to synchronize the cell cycle and seeded on 96-well-plates at an optimum cell density of 40,000 cells/ml. Proliferation activity was photometrically assessed after 24, 48, and 72 hours at a wavelength of 570 nm (Tecan). For total cell counts rat SMC cells (A10) derived from thoracic aorta of DB1X embryonic rats were cultured in 24-well plates (5x10^3/well) on day 0. After 4 hours the cells were transfected using recombinant adenovirus Ad_ABRA and Ad_LacZ (0.5x10^5 ifu/well). For 3 days duplicate wells (n=4) were harvested and counted using a Neubauer counting chamber.

Cyclic mechanical stretch was applied as described. RASMC were plated on 6-well silicone Elastomer plates coated with collagen type I (Bioflex; Flexcell, Hillsborough, NC). The cells were exposed to continuous cycles of stretch and relaxation (1.0 Hz) using the Flexercell Strain Unit FX-3000 (Flexcell) for the indicated times; a maximum of 10% radial stretch of the membrane was applied.
RNA isolation, quantitative real time PCR (qRT-PCR) and microarray analysis

7 days after surgery total RNA was isolated (RNeasy Mini kit, Qiagen) from dissected collaterals or other organs. After treatment with DNase-I (Turbo DNAfree, Ambion), microarray analysis was performed essentially as described. cDNA was synthesized according to Superscript II reverse transcriptase protocol (Invitrogen) using 300 ng total RNA and 200 ng random nonamer oligonucleotides (NEB). Gene-specific RT-PCR primers were selected using FastPCR software (Institute of Biotechnology, University of Helsinki, Finland). qRT-PCR was performed in a 25 µl reaction, 96-well format (1.0 µl cDNA (1:20); 200 nM each primer; 1X IQ SYBR Green Super Mix (BioRad) using an iCycler real time PCR system (BioRad). Samples were measured in triplicate, with a minimum of two independent experiments. The relative amount of target mRNA normalized to 18S RNA was calculated as previously described.

For mRNA profiling RNA samples (AV-shunt and sham) were linearly amplified for two cycles to average yields of 11 µg cRNA (average l 260/280 ratio 1.96) and subsequently labeled with Cy3- and Cy5 dyes (Amino Allyl MessageAmp™ aRNA Kit, cat#1752, Ambion) for Microarray analysis. Each sample pair of AV-shunt and control collaterals (1 µg cRNA per Cy-dye) was hybridized in duplicates according to the dye-swap design for 16h at 40°C (25% de-ionized formamid; 20 mg yeast tRNA; Hyb buffer, Amersham). Microarray analysis was performed essentially as described.

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. 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. 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. The following antibodies were used: anti-*Abra* (generated in chicken by Eurogentec; Peptides corresponding to amino acids 1-15 and 273-287 rat *Abra* sequence (Acc.Q8K4K7), rabbit anti-RhoA (abcam), rat anti-Ki67 tec3 (Dako), mouse anti α-actinin (Sigma), Cy3-conjugated anti-α-smooth muscle actin (*Actg2*) (Sigma), FITC-conjugated goat-anti-chicken IgG (Invitrogen), CF488-conjugated goat anti-rabbit (Biotrend), Cy3-conjugated donkey anti-rat (chemicon). Nuclei were stained with Draq5 (Alexis Biochemicals) and F-actin was stained with FITC-conjugated Phalloidin.
(Sigma-Aldrich), G-actin was stained with Alexa 595 labeled DNase I (Invitrogen). Sections were viewed with a confocal microscope (Leica TCS SP).

**Statistical Analysis**

All values are expressed as mean ± s.e.m.. Two treatment groups were compared by the unpaired Student´s 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.
**Figure legends**

**Table T1:** PCR primer sequences

**Table T2:**
Microarray data revealed that three functional groups of genes are over represented among 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 control collaterals. Using Gene Set enrichment analysis (GSEA) \(^{13}\), Kyoto Encyclopedia of Genes and Genomes (KEGG, \[http://www.genome.jp/kegg/\]) and data mining, an enrichment of transcripts displaying a role in remodeling of the actin cytoskeleton, an activation of the Rho/ROCK-pathway, the calcium signaling pathway was observed. Interestingly, a number of cardiac genes are also up-regulated.

**Figure S2:** Abra expression in RASMCs exposed to cyclic stretch
Rat aortic smooth muscle cells (RASMC), which were exposed to cyclic stretch (1.0 Hz, 10% elongation) for 0.5 to 24 h, did not show an altered Abra mRNA abundance.

**Figure S3:** Antibody test: A. Serial sections of rat quadriceps muscle after shunt treatment. (Abra: green; αSMA: red; Nuclei: blue; scale bars: 30 µm.). Left panel shows immunoabsorption using Abra peptide in order to test antibody specificity. B. Western Blot of Abra overexpressing COS1 cells (+); untransfected control cells (-). C. In longitudinal sections of skeletal muscles stained for Abra and α-Actinin confirmed partial overlap of Abra with the Z-line.

**Figure S4:** Porcine vascular cells were transduced with Ad_Abra or Ad_LacZ. Immuncytochemistry and Western Blot were performed as transduction controls (scale bars 40 µm).

**Figure S5:**
Confirmation of increased proliferative activity in smooth muscle cells. Rat A10 cells were transduced with Ad_Abra or Ad_LacZ and cells were counted on day 1, 2, and 3.
Figure S6: Abra Promoter analysis

(A) Genomatix software (Genomatix Software GmbH, Germany) was used to analyse the Abra promoter region 500 bp upstream of the transcriptional start site. The sequence matching MEF2 and ATF4 transcription factor binding sites were highly conserved among the investigated promoter sequences, which signifies transcriptional control of Abra by these transcription factors. (B) In silico analysis (Genomatix Software GmbH, Germany) of the rat Abra promoter revealed a downstream regulator element site (DRE site). Upon binding of Ca^{2+}, the downstream regulator element-antagonist modulator (Dream) is released from the DRE site, which leads to a de-repression of the promoter.
References


12. Rodriguez M, Cai WJ, Kostin S, Lucchesi BR, Schaper J. Ischemia depletes dystrophin and inhibits protein synthesis in the canine...

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<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Abra</td>
<td>tgc tgc cag gat taa acg ccc ct</td>
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<tr>
<td>Smoothelin</td>
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**Table 1**

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<th>Gene Name</th>
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<td>Gelsolin</td>
<td>Csln</td>
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<td>Cell division cycle 42 homolog (S. cerevisiae)</td>
<td>Cdc42</td>
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<td>Fibronectin 1</td>
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<td>Actin, alpha 4</td>
<td>Actn4</td>
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<td>Myosin light chain kinase 2, skeletal muscle</td>
<td>Mylk2</td>
<td>2.0</td>
<td>0.04915</td>
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**Rho Pathway**

| Physa ras-related homolog A2           | Rhoa        | 2.0       | 0.01744 |
| Obscurin                               | Obscn       | 4.0       | 0.04000 |
| TC10-like Rho GTPase                   | Rhog        | -2.2      | 0.01417 |
| T-cell activation GTPase activating protein | Tagap    | -2.3      | 0.04926 |
| Rho GDP dissociation inhibitor (GDI) gamma | Armgdg   | -3.8      | 0.00332 |

**Calcium signaling pathway**

| Ryanodine receptor 1 (skeletal)        | Ryr1        | 4.7       | 0.00390 |
| ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 | Atp2a1     | 6.4       | 0.00052 |
| transient receptor potential cation channel, subfamily V, member 4 | Trpv4      | 3.45      | 0.03390 |
| Glutamate receptor, ionotropic, NMDA2D | Grin2d     | 10.1      | 0.00022 |
| Phospholipase C, beta 3                | Plcb3       | -4.8      | 0.02394 |
| Adenylate cyclase 2                    | Adcy2       | 4.6       | 0.00188 |
| Solute carrier family 25 member 4      | Slc25a4     | 8.1       | 0.00014 |
| Solute carrier family 25 member 5      | Slc25a5     | 3.2       | 0.02464 |

**Cardiac genes**

| similar to troponin C, cardiac muscle - mouse (LOC290561), mRNA | LOC290561 | 11.6 | 0.00059 |
| similar to cardiac leiomodin (LOC296935), mRNA | LOC296935 | 11.3 | 0.00866 |
| myosin, heavy polypeptide 7, cardiac muscle, beta (Myh7), mRNA | Myh7 | 9.2 | 0.00084 |
| similar to actin, alpha, cardiac (LOC290654), mRNA | LOC290654 | 8.4 | 0.00015 |
| similar to Ryanodine receptor 2 (Cardiac muscle-type) | LOC361535 | 6.5 | 0.00018 |
| ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 | Atp2a1 | 6.4 | 4.30E-06 |
| Adenylate cyclase 1 | Adcy1 | 6.4 | 0.02466 |
| ATPase, Ca++ transporting, cardiac muscle, slow twitch 2 (Atp2a2) | Atp2a2 | 2.6 | 0.02009 |
| troponin T2, cardiac (Tnnt2), mRNA | Tnnt2 | 2.1 | 0.02257 |
| similar to Myosin regulatory light chain 2, ventricular-cardiac muscle isoform (MLC2) | LOCC307234 | 1.7 | 0.03817 |
| similar to Myosin regulatory light chain 2, ventricular-cardiac muscle isoform (MLC2) | LOC293925 | 1.5 | 0.03787 |
| Tropomycin C, cardiac/slow skeletal (predicted) | BC204478 | 1.5 | 0.01278 |
| Tropomycin C, cardiac/slow skeletal (predicted) | BC395510 | 1.3 | 0.00588 |
| Ankyrin repeat domain 1 (cardiac muscle) | Ankrd1 | 1.3 | 0.03147 |

**Table 2**
Figure S1
Abra mRNA Expression

Cyclic stretch

Figure S2
Figure S3
figure S4
A10 cells

number of cells / well (x1000)

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** figure S5 **
Figure S6

A

B