Distinct Cellular Mechanisms Underlie Smooth Muscle Turnover in Vascular Development and Repair

Urmas Roostalu¹, Bashar Aldeiri¹, Alessandra Albertini¹, Neil Humphreys², Maj Simonsen-Jackson², Jason KF Wong¹,³, Giulio Cossu¹

¹Manchester Academic Health Science Centre, Division of Extracellular Matrix and Regenerative Medicine, Faculty of Biology, Medicine and Health, University of Manchester, UK; ²Transgenic Core Research Facility, Faculty of Biology, Medicine and Health, University of Manchester, UK, and; ³Plastic Surgery Department, University Hospital South Manchester Trust, Wythenshawe Hospital, Manchester, UK.

Running title: Smooth Muscle Lineage Continuity in Arterial Wall

Subject Terms:
Animal Models of Human Disease
Cardiovascular Surgery
Developmental Biology
Gene Expression and Regulation
Smooth Muscle Proliferation and Differentiation

Address correspondence to:
Dr. Urmas Roostalu
University of Manchester
Michael Smith Building
Oxford Road, M13 9PL
Manchester, UK
urmas@roostalu.info

In October 2017, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13 days.

DOI: 10.1161/CIRCRESAHA.117.312111
ABSTRACT

Rationale: Vascular smooth muscle turnover has important implications for blood vessel repair and for the development of cardiovascular diseases, yet lack of specific transgenic animal models has prevented its in vivo analysis.

Objective: To characterize the dynamics and mechanisms of vascular smooth muscle turnover from the earliest stages of embryonic development to arterial repair in the adult.

Methods and Results: We show that CD146 is transiently expressed in vascular smooth muscle development. By using CRISPR-Cas9 genome editing and in vitro smooth muscle differentiation assay we demonstrate that CD146 regulates the balance between proliferation and differentiation. We developed a triple-transgenic mouse model to map the fate of NG2^+ CD146^+ immature smooth muscle cells. A series of pulse-chase experiments revealed that the origin of aortic vascular smooth muscle cells can be traced back to progenitor cells that reside in the wall of the dorsal aorta of the embryo at E10.5. A distinct population of CD146^+ smooth muscle progenitor cells emerges during embryonic development and is maintained postnatally at arterial branch sites. To characterize the contribution of different cell types to arterial repair we used two injury models. In limited wire-induced injury response existing smooth muscle cells are the primary contributors to neointima formation. In contrast, microanastomosis leads to early smooth muscle death and subsequent colonization of the vascular wall by proliferative adventitial cells that contribute to the repair.

Conclusions: Extensive proliferation of immature smooth muscle cells in the primitive embryonic dorsal aorta establishes the long-lived lineages of smooth muscle cells that make up the wall of the adult aorta. A discrete population of smooth muscle cells forms in the embryo and is postnatally sustained at arterial branch sites. In response to arterial injuries existing smooth muscle cells give rise to neointima, but upon extensive damage they are replaced by adventitial cells.

Keywords: CD146, YAP1, smooth muscle turnover, development of aorta, arterial repair, smooth muscle differentiation, smooth muscle progenitor cells, developmental biology, arterial injury, transgenic mice.

Nonstandard Abbreviations and Acronyms:

NG2 Neural/Glial Antigen 2
SCA1 Stem Cell Antigen 1
SFA Superficial Femoral Artery
SMMHC Smooth Muscle Myosin Heavy Chain
TAGLN Transgelin
VSMC Vascular Smooth Muscle Cell
YAP1 Yes Associated Protein 1
INTRODUCTION

Vascular smooth muscle cells (VSMCs) develop early in the embryo and are essential in regulating vascular tone and strengthening blood vessel walls. The descending aorta arises by fusion of primordial aortae at the midline and as the development proceeds branches out to supply blood to the growing organs. VSMCs are recruited from the surrounding tissues and as such their origin varies depending on the location of the blood vessel \(^1\), \(^2\). The mechanisms by which VSMCs are recruited and the dynamics of their proliferation are still poorly understood.

Rapid regeneration of smooth muscle coverage is essential for successful repair of vascular injuries. The extent in which the regeneration relies on the proliferation of existing VSMCs and on the recruitment of diverse progenitor cells has been hotly debated \(^1\), \(^3\)-\(^5\). VSMCs can in vitro convert from differentiated state into proliferative phase in a process known as phenotypic switching \(^6\). Several studies have demonstrated VSMC proliferation and contribution of dedifferentiated VSMCs to neointima formation following vascular injury \(^7\)-\(^12\). Thus, extensive indirect evidence supports the capacity of already differentiated VSMC to re-enter cell cycle. Nevertheless, direct in vivo analysis of this process has been complicated due to the lack of truly VSMC-specific inducible transgenic models. Furthermore, accumulating evidence suggests a great deal of variability in the capacity of VSMCs to respond to injuries \(^13\). The underlying cause of this variability has remained unknown.

Various stem and progenitor cells have been described around arteries. Stem cell antigen 1 (SCA1) marks a large proportion of adventitial cells that are capable of giving rise to VSMCs following transplantation to experimental vein grafts \(^14\). Similar cells have been identified in human arteries \(^15\), \(^16\). Adventitial CD34\(^+\) progenitor cells in rat had minimal role in neointima formation, but did provide VSMCs to the outer medial layer of the artery \(^17\). A population of adventitial cells expresses mesenchymal stromal cell markers and can contribute to vascular calcification \(^18\)-\(^20\). Lack of cell type-specific marker genes and transgenic models has prevented the construction of a developmental hierarchy of vascular progenitor cells. The diversity of the vascular tree may create different local niches for progenitor cells, influenced by restricted signaling gradients and physical properties. This so far uncharacterized variability may lie behind the variegated response of the vasculature to injuries.

We found that immature VSMCs are marked by high expression of CD146 that controls their maturation. Using a novel transgenic mouse model we show that adult aorta VSMCs originate from embryonic dorsal aorta resident progenitor cells with limited contribution from surrounding stem or progenitor cells in the following growth period. We describe here a distinct self-renewing population of CD146\(^+\) progenitor cells at arterial branch points. In response to minor injuries to the artery VSMCs give rise to the majority of neointima cells, whereas in severe injuries early VSMC death occurs and is followed by smooth muscle differentiation of adventitial progenitor cells.

METHODS

All developed research models and methods are available upon request from the corresponding author. Detailed methods are described in the Online Supplement. For adult mice 2 mg (0.1 mg/g of body weight) of tamoxifen (TAM) (in corn oil) was injected intraperitoneally for 5 consecutive days. For neonatal labelling 0.25 mg was injected subcutaneously for 3 days (P6-P8). For embryonic labelling 3 mg of TAM was injected together with 1.5 mg progesterone into the intraperitoneal cavity of pregnant female. For long term lineage-mapping mice were delivered at P20 by caesarean section and fostered. Embryos were fixed in 4% paraformaldehyde (PFA) for 5 h. For postnatal stages PFA was perfused intracardially, followed by 3 h incubation of isolated organs in PFA.
Vascular injury was performed under Home Office License PPL 70/8686 according to the UK Animals (Scientific Procedures) Act (1986). Mice were anesthetized with 4% Isoflurane with 2 l/min O2. The operation site was shaved and cleaned with 0.2% chlorhexidine. An incision was made along the medial aspect of the leg to expose the superficial femoral artery (SFA). The artery was separated from the femoral nerve and vein controlled using 2-V Acland clamps (S&T, Switzerland) around the site of vascular division. For the super-microanastomosis model the artery was repaired at 40 x magnification using an intravascular stenting technique and 12/0 nylon microsutures (S&T, Switzerland). Vessel patency was checked for good flow through the anastomosis and after repair. Skin and soft tissues were closed with 8/0 nylon sutures (Ethicon, UK). Wire-induced injury was performed as described. 3 mm of wire length was inserted into the artery, moved back and forth 10 times and retrieved. The arterial wall was repaired by two 12/0 nylon sutures.

Zeiss Axio Imager M2, Zeiss Axio Zoom and Zeiss Axio Observer.Z1 microscopes were used with Zeiss Zen 2 software. Leica SP5 and SP8 confocal microscopes were used with Leica Application Suite software. Images were acquired at 2 times line average and 4-6 times frame average, using sequential scanning mode. Cells were counted in ImageJ 1.51n. Cell counting on histological samples was blinded (counting on Hoechst channel before immunohistochemistry). Statistical analysis was carried out in GraphPad Prism 7. Dunnett’s test was used to analyze statistical significance of the differences between multiple groups. Unpaired two-tailed t-test was used to compare two data sets.

RESULTS

Cell adhesion molecules regulate diverse developmental processes. We searched for genes that can uniquely identify developing VSMCs and focused on the expression dynamics of NG2 (neural/glial antigen 2; Cspg4) and CD146 (Mcam – melanoma cell adhesion molecule). NG2 is expressed by VSMCs, glia, myocytes, adipocytes and chondrocytes. CD146 is expressed by endothelial cells, VSMCs and mesenchymal stromal cells. We found that before the primordial aortae have fused together, at E8.5, they lack NG2+ VSMCs while CD146 expression is limited to endothelium (Figure 1A, Online Figure I A). By E10.5 the fusion process is largely completed and VSMCs can be detected by the expression of smooth muscle alpha actin (SMA), NG2 and CD146. While NG2 expression is maintained in VSMCs at later time points, the expression of CD146 shows a marked decrease in the descending aorta by E16.5. At the same time it is maintained in microvascular pericytes and smaller branches of the artery. In adult mouse descending aorta only scattered expression of CD146 is evident in VSMCs (6.1+/-2.8% (SD); n=5; Figure 1B). The development of the abdominal aorta lags behind thoracic aorta (Figure 1A). At E10.5 it has not completed the fusion process, although it has acquired CD146+ VSMCs. At E16.5 CD146 expression is still uniformly maintained in the abdominal aorta. These results indicate that the transient expression of CD146 marks undifferentiated VSMC progenitors.

Considering the temporally limited expression of CD146 in VSMC development we next addressed its functional importance in the differentiation process. We used CRISPR-Cas9 to delete CD146 in 10T1/2 cells, which serve as an in vitro model for VSMC differentiation upon TGFβ1 exposure. Wildtype 10T1/2 cells upregulate transgelin (TAGLN/SM22α), SMA and smooth muscle myosin heavy chain (SMMHC, Myh11) after 48 h exposure to TGFβ1 (Figure 1C-E, Online Figure I B, Online Tables I-II). Importantly, both CD146 knockout cell lines (C149, C164) showed high level spontaneous expression of characteristic VSMC markers even in the absence of TGFβ1, comparable to unedited cells exposed to differentiation conditions. Upon exposure to TGFβ1 the mutant cells showed significantly higher expression levels of SMA and TAGLN than unedited cells. Both mutant cell lines proliferated less than
wild type cells (Figure 1C, Online Figure I C, Online Tables I-II). These results demonstrate that CD146 regulates the balance between VSMC differentiation and proliferation.

Our data indicates that developing VSMCs can accurately be identified by NG2 and CD146 co-expression, whereas other cell types express either one or the other gene separately. We developed a novel VSMC-specific lineage tracing mouse model that relies on sequential activation of two recombinases. We fused tdTomato red fluorescent protein via P2A self-cleaving peptide to nuclear localized flippase (FLPO) (Figure 2A) and insertedlox2272 flanked STOP cassette upstream of this sequence. We identified 12 kb region upstream of CD146/Mcam in ENCODE datasets carrying epigenetic signatures of active enhancers (Online Figure II A). A CCCTC-binding factor enriched region at its 5′ end defines a putative insulator. We synthesized this DNA together with the first intron of CD146/Mcam in ENCODE datasets carrying epigenetic signatures of active enhancers (Online Figure II A). A CCCTC-binding factor enriched region at its 5′ end defines a putative insulator. We synthesized this DNA together with the first CD146/Mcam intron, inserted tdTomato-P2A-Flpo cassette in place of the first exon and placed HS4 insulator on the 3′ side. The linearized DNA was injected to mouse zygotes to generate CD146-T2F transgenic line. When this line is crossed to NG2-CRE-ERTM strain, the STOP cassette is removed by CRE after tamoxifen (TAM) administration, initiating the expression of tdTomato and FLPO only in CD146+NG2+ VSMCs (Figure 2B). By crossing the double transgenic line to FLPO reporter RCE-FRT, GFP expression is triggered by ubiquitous promoter. As a result CD146+NG2+ VSMCs are marked by tdTomato and GFP, whereas their CD146− progeny is labelled only by GFP.

We verified the specificity of the double-transgenic line by analyzing the expression of tdTomato relative to native CD146 and NG2. TAM administration at E10.5 led to tdTomato labelling of VSMCs in the dorsal aorta and femoral artery by E12.5 (Figure 2C, D). Since endothelial cells express only CD146 and not NG2, they remained unlabeled (Figure 2C). Likewise, limb chondrogenic cells express only NG2 and not CD146, thus lacking tdTomato expression and proving the specificity of the developed transgenic strategy (Figure 2D, E). Early microvasculature lacks NG2+ pericytes and tdTomato labelling (Figure 2E). TAM injection at E14.5 and analysis at E16.5 revealed labelling of CD146+NG2+ VSMCs in the thoracic aorta, but not in the vena cava that lacks NG2+ VSMCs (Figure 2F). TdTomato expression was evident in pulmonary arteries, abdominal aorta, renal arteries and microvascular pericytes (Figure 2G-J, Online Figure II B), indicating that the transgene marks VSMCs arising independently of different origins and recapitulates the expression patterns of CD146 and NG2. Embryonic VSMCs express platelet derived growth factor β that is much more widely spread in the mesoderm (Online Figure II C). There was no labelling in the lymphatic vessels (Online Figure II D). We calculated recombination efficiency by using double antibody staining (NG2/CD146) in the dorsal aorta at E12.5 and found it to be 62% (+/-5.2%; SD, n=6), whereas it was 29% (+/- 8.3%; SD, n=6) in the kidney microvasculature at E16.5. An inevitable problem with conditional transgenic models is the leakiness of the STOP cassette. In this case STOP read-through would label endothelial cells that express CD146 and not NG2. We used FACS to calculate GFP marked endothelial cells and found that in most organs less than 0.2% of endothelial cells expressed GFP, with 3.9% (+/-1.6%; SD, n=3) of being labelled in the skeletal muscle (Online Figure II E-F). These results indicate only limited leakiness.

It is not known when the VSMCs that make up the mature aortic wall populate the vascular niche. We used triple transgenic strain CD146-T2F:NG2-CRE-ERTM:FRT-GFP to reveal the turnover of CD146+NG2+ VSMCs. Pulse-chase from E10.5 to E15.5 labelled 29% of all the VSMCs in the thoracic aorta by tdTomato and GFP and 13.5% only by GFP (Figure 3A, C). In the abdominal aorta the respective proportions were 43% and 12.5% (Figure 3B, C). This indicates that VSMCs maintain CD146 expression and immature phenotype during embryonic development. Fate-mapping analysis spanning late embryonic and fetal stage (E14.5 to E17.5) led to reduced labelling, with 3.3% of thoracic aorta VSMC being tdTomato‘GFP’ and 4.8% tdTomato GFP+. The transgene expression activity proves cranio-caudal VSMC differentiation, since in fetal stage still 14.3% of VSMC in the abdominal aorta were tdTomato‘GFP’ and 7.8% tdTomato GFP+ (Figure 3C-E; Online Figure III A-B). Mice grow rapidly in size and their body weight increases by nearly fivefold in the first 20 days after birth. This juvenile growth period was not

DOI: 10.1161/CIRCRESAHA.117.312111
coupled with reactivation of CD146 expression in the aorta. At P22 less than 1% of VSMCs in the aortae were marked by tdTomato and less than 8% by GFP (TAM injection P6-P8, analysis at P22; Figure 3C, F-H; Online Figure III C). Transgene expression was still efficiently triggered in smaller arterioles and microvasculature (Online Figure III D). Limited labelling was evident when TAM was injected in adult mice (5 injections after P35, analysis at P54) (Figure 3C, Online Figure III E). We detected global organ level decrease in tdTomato labelling in adult skeletal muscles and lung, suggesting that microvascular pericytes lose CD146 with aging (Figure 3I, Online Figure III F). To confirm that the loss of transgene labelling in mature VSMCs is not caused by limited activity of NG2-CRE-ERTM transgene, we crossed NG2-CRE-ERTM line to Rosa26-tdTomato reporter. TAM administration in these mice (5 injections after P35, analysis at P54) led to prevalent tdTomato expression in the abdominal aorta VSMCs (58.6 +/- 3.4%; SD, n=3) (Online Figure III G), proving that the decrease in CD146 expression underlies the downregulation of the transgene in mature VSMCs.

CD146+ cells make up the majority of embryonic aorta VSMCs and the transgene does not label neighboring adventitial cells (Figure 2C; 3A, B). We asked, whether the progeny of the embryonic CD146+ VSMCs is maintained in the aorta or replaced during development and postnatal growth. We provided a single TAM pulse at E10.5 and analyzed the aortic wall at P35. We found that the majority of VSMCs were labelled with GFP (79% in the abdominal aorta, 68% in the thoracic aorta) (Figure 3C, J-K). We conclude that the origin of adult descending aorta VSMCs can be traced back to progenitor cells that exist in the aorta at the time of its development at E10.5.

By analyzing embryonic arterial vasculature we noticed high expression of CD146 in VSMCs at aortic branching sites (Figure 4A). While TAM administration at E14.5 led to limited labelling of VSMCs in the aortic wall at E17.5, it still labelled VSMCs at aortic branching sites (Figure 4B). Remarkably, these branch site associated cells maintain high CD146 expression even in the mature aorta (Figure 4C) and consequently are also labelled by tdTomato (Figure 4D, Online Figure IV A). We found tdTomato+ branch site associated cells in adult mice even after a single TAM pulse at E10.5. This indicates that these cells originate early in embryonic development and have long-term self-renewal capacity (Figure 4E). In order to prove the specificity of the transgene, we generated a second transgenic mouse line from an independent founder and also detected high tdTomato labelling in the branch site associated progenitor cells (Online Figure IV B). In contrast to the aorta we found that smaller arteries, like the SFA and mesenteric arteries are efficiently marked by tdTomato in adulthood (Figure 4F-H). When TAM was injected in adult mice (5 doses in 3 month old mice, analysis 2 weeks later) 38.3% of VSMCs in the SFA were tdTomatoGFP+ and 11.6% tdTomatoGFP-. Still, even in the SFA and the mesenteric artery tdTomato was more enriched at arterial branching sites (Figure 4I-J). These data show that high CD146 expression identifies a unique cell population at arterial branching sites.

We next focused on the functional significance of CD146 expression and the potential role of branch site associated NG2’CD146+ cells. We first hypothesized that maintained CD146 expression may correlate with increased cell turnover at arterial branching sites and in smaller arteries. We carried out a thorough quantification of Ki67 staining at different developmental stages (n=4; average 566 VSMCs analyzed per mouse). These analyses revealed that 93% of VSMCs are proliferative in the aorta at E10.5, whereas by E16.5 there is a significant drop to 30% and 25.5% in the abdominal and thoracic aorta respectively (Figure 5A, Online Figure V A). This highly proliferative phase coincides with the period when the cell lineages that are maintained to adulthood first emerge. The decrease in cell proliferation occurs earlier in the thoracic aorta, supporting cranio-caudal aorta maturation. At P10 18% of abdominal aorta VSMCs are proliferative, whereas cell proliferation becomes rare (0.4% in abdominal, 0.8% in thoracic aorta) in 3 month old mice. We studied VSMC apoptosis by staining for active caspase 3 (CASP3). We analyzed 700 VSMCs in the abdominal and thoracic aortae in 4 mice (5600 VSMCs in total in the descending aortae) but could not detect any apoptotic VSMCs, although other cells in the surrounding tissues were labelled (Online Figure V B). These results support limited VSMC turnover in the mature aorta.
We then analyzed aortic branch sites (intercostal artery branching sites from the thoracic aorta) but found no overall significantly higher cell proliferation: 3.7% (SD 3.03) of branch site associated VSMCs are KI67+ (Figure 5A). There was large variability in cell proliferation across postnatal aortic branching sites, with high proliferative activity in some but no KI67+ cells in others (Figure 5A-B). This suggests that aortic branch sites do not maintain a constantly elevated cell turnover, yet cell proliferation here may occur in isolated phases. We found that only 0.6% of VSMCs in the SFA were marked by KI67, which is not significantly different from the aorta (Figure 5A, Online Figure V A). Consequently, sustained CD146 expression does not necessarily correlate with higher cell proliferation rate.

CD146+NG2+ cells cluster in cushion-like structures that extend to the aortic lumen at branching sites. These cells represent immature smooth muscle cells that express low levels of SMMHC (Figure 5C). They share also some morphological similarities with pericytes (small size, cellular processes). VSMCs respond to $\alpha_1$-adrenergic receptor agonist phenylephrine by intracellular increase in Ca$^{2+}$ concentration, whereas pericytes often do not show this property. We found that cells at aortic branch site respond to phenylephrine by transient yet modest increase in intracellular Ca$^{2+}$ concentration, indicating that despite expressing low levels of VSMC contractile proteins they are still capable of responding to vasoconstrictive stimuli (Figure 5D-E). Arterial branching sites are exposed to elevated blood flow turbulences and smaller arteries have to respond to mechanical stretch. We hypothesized that CD146 may, in addition to controlling VSMC maturation, be involved in cellular adhesion to stabilize the vasculature. We confirmed that CD146 loss leads to reduced adhesion of 10T1/2 derived VSMCs (Figure 5F-G, Online Figure V C). One of the best characterized mecano-transduction pathways is centered on YAP1 (Yes Associated Protein 1) signaling, which intriguingly is one of the few known transcriptional regulators of CD146. YAP1 mediates VSMC phenotypic switching and is downregulated in VSMC maturation. This prompted us to study YAP1 expression in the aorta. We found that whereas mature VSMCs express low levels of active phosphorylated YAP1 it is highly expressed at arterial branching sites, providing an insight into how CD146 is maintained at these locations (Figure 5H).

The relative contribution of VSMCs and adventitial cells to vascular repair has remained uncertain possibly due to the use of non-specific transgenic models and variations in injury methods. Since in our triple-transgenic model only VSMCs are labelled in adult mouse SFA it enables precise fate mapping of VSMCs following vascular injury. We first used established wire-induced injury model that causes damage to the tunica intima. This model has been widely utilized to study the formation of neointima, which we confirmed here (Figure 6A-B). We induced transgene expression in adult mice (at 2-3 months, 5 daily TAM injections prior to surgery), inflicted arterial injury and following 3 weeks analyzed the origin of neointima cells. We found that 51.7% of neointima cells were marked by GFP and tdTomato whereas 4.8% expressed only GFP (Figure 6C-E). Considering recombination efficiency our results indicate that the majority of neointima cells originate from VSMCs and maintain CD146 expression.

We next developed a clinically relevant severe injury and repair model of distal arteries. The SFA with a luminal diameter of 0.1-0.3 mm was transected proximal to the popliteal branching site and repaired in a super-microanatomosis fashion using six interrupted 12/0 sutures (Figure 6F, Online Figure VI A-B). The complete transection nature of the injury inflicts significant transluminal damage across all layers of the arterial wall similar to what is seen in human arterial injury and repair. All operated mice (n=11) recovered well following the surgery and we did not observe any difference in limb activity up to the time of tissue retrieval. We analyzed the repair process at early (48 hours, n=5) and late (2 weeks, n=6) time points. We detected partial thrombus formation in all mice at 48 hours following the repair (Figure 6G). Yet, at 2 weeks no occlusion was evident in 4 out of 6 mice (Figure 6H). Remarkably, the SFA wall around the site of anastomosis was devoid of GFP+ VSMCs (Figure 6I-J). Intact endothelium was present in the operated SFA and GFP+ microvascular pericytes were detected around the site of anastomosis (Figure 6I, Online Figure VI C). Severe vascular injuries are known to cause VSMC apoptosis during the first 2 hours. We also confirmed loss of VSMC at 48 hours after SFA anastomosis (Online Figure VI D-E).
death has been proposed to be silent in mice and can leave behind an acellular yet functional vascular wall scaffold. We therefore analyzed whether re-cellularization and regeneration occurs following SFA super-microanastomosis.

We constructed detailed spatial map of the SFA relative to the site of anastomosis (Figure 7A). In zone 1, distal to the site of injury, the SFA had normal morphology and GFP+TAGLN+ VSMCs (Figure 7B). Zone 2 encompasses the site of anastomosis and area immediately proximal to it, where GFP+ VSMCs were replaced during regeneration. Importantly, 2 weeks following the surgery the vascular wall had an expanded TAGLN+ cell layer. These cells showed scattered expression of SMMHC, indicative of terminal VSMC differentiation. Yet, they were not labelled by GFP and thus did not arise from existing VSMCs (Figure 7B, C). Zone 3 forms an intermediate area further cranially, where we detected disorganized VSMC layer and GFP+ neointima (Figure 7B). In zone 4 the arterial morphology was indistinguishable from healthy artery. These data prove that in severe injury the pre-existing VSMCs are replaced by a new layer of VSMCs that do not arise from existing VSMCs.

To gain an insight into the origin of the cells participating in arterial repair we analyzed cell proliferation in the above described zones. We found that in zone 3, 23% of the GFP+VSMCs were proliferative (Figure 8A, D). Around the site of anastomosis, where the existing VSMC were replaced, we detected expansion in the cell population characterized by adventitial markers CD44+, SCA1+, and CD34+ (Figure 8B-C, F-G, Online Figure VII A-D). Most notable is the induction of CD44 staining, which is rarely evident in the healthy control artery (Figure 8B, F, Online Figure VII A). We did not detect such intrusion of the intimal layers by adventitial cells following wire-induced injury (Online Figure VII E-F). The adventitial cells were frequently proliferative 2 weeks after SFA anastomosis: 13.3% of SCA1+ and 26.2% of CD44+ cells stained for Ki67 (Figure 8B, C, E). These results collectively suggest that new VSMCs may arise from SCA1+, CD44+ and CD34+ progenitor cells at the site of anastomosis. We conclude that the degree of VSMCs contribution to vascular repair depends on the severity of injury. While VSMCs are capable of repairing milder intimal injuries, they fail short when transluminal injury takes place.

**DISCUSSION**

VSMCs have major significance in cardiovascular diseases, yet their turnover has remained poorly characterized due to the lack of specific transgenic models. Many cell types are capable of VSMC differentiation and hence uncertainty has persisted on the origin and heterogeneity of VSMCs of the aorta. By developing a novel VSMC specific lineage tracing model we demonstrate that adult aorta VSMCs originate from progenitor cells residing in the wall of the aorta already in early embryonic development (Figure 8H). Extensive VSMC proliferation occurs in embryonic period but only limited cell turnover take place in the adult, which is consistent with VSMCs losing their intrinsic proliferative capacity after birth. Mature VSMCs in the mouse aorta have an estimated half-life of 270-400 days. These data collectively indicate that VSMCs of the aorta represent very long-lived and self-maintaining cell lineages that arise in the early embryo.

We show that CD146 is transiently expressed in VSMCs in the embryonic development of the aorta, whereas smaller arteries maintain its expression to adulthood. We found that CD146 regulates the balance between VSMC proliferation and differentiation. CD146 is a co-receptor for PDGFRβ, VEGFR2, binds WNT1 and 5A, several other ligands, mediates diverse signaling pathways and cell adhesion. We propose that CD146 fine tunes different signaling cascades to regulate contractile protein levels in VSMCs, thereby enabling more flexible cell shape in developing tissues and arteries. We show that it is also required in VSMC adhesion and may thus strengthen smaller arteries that are affected by movement.
We describe here a hitherto unrecognized population of immature VSMCs that emerges in embryonic development and is confined to aortic branching sites after birth. Since at least 1950s it has been known that intimal cells form cushion-like structures at arterial branching sites \(^{55, 56}\). These sites have in recent years gained considerable attention as they appear to be more susceptible to atherosclerotic lesions \(^{57}\).

However, up to now no single marker gene has been identified for arterial branching sites and their cellular composition has remained unknown. We show that the unique intimal VSMC population at arterial branching sites is marked by pYAP1 and CD146 expression. As CD146 prevents VSMC differentiation then these branch site cells express low levels of SMMHC. Yet, they can still respond to contractile stimuli. Considering their undifferentiated state these cells may contribute to new VSMCs in the growth of lateral branches. However, their primary function in the adult is likely to provide structural support to the arterial branching sites.

Finally, we demonstrate that different cell populations underlie vascular wall remodeling following injury. Various cells have been proposed to contribute to neointima formation and controversies have persisted largely due to the lack of VSMC specific fate mapping models. All VSMC contractile proteins and their regulators (SMMHC, SMA, TAGLN) are also expressed in fibroblasts that may arise from different origins and also populate injury sites \(^{58-60}\). We show that in response to limited injury local VSMCs are the primary contributors to neointima formation, supporting some previous conclusions \(^{7, 8}\). Severe injuries lead to early VSMC death, leaving behind extracellular matrix scaffold in arterial wall \(^{48}\). We found that this matrix is repopulated by cells that differentiate into new VSMCs, yet these cells do not originate from existing VSMCs around the injury site, but rather from adventitial cells. Adventitial cells marked by SCA1, CD44, CD34 and GLI1 are capable of VSMC differentiation \(^{14, 17, 18, 61, 62}\). We propose that differentiated VSMCs cannot respond rapidly to migrate towards injury and their contribution is therefore limited to local microenvironment.

By using in vivo lineage tracing we have revealed here the vascular turnover of smooth muscle cells throughout mouse development and postnatal growth as well as in arterial injury repair. We identified a population of smooth muscle progenitor cells that resides in a specific niche at arterial branch sites.

ACKNOWLEDGEMENTS
We thank M. C. Jackson and the Flow Cytometry facility, P. March and the Bioimaging facility, G. Bako, P. Walker and the Histology core facility (Faculty of Biology, Medicine and Health, Manchester). We are grateful to E. Owen for mouse colony management.

SOURCES OF FUNDING
U.R. was supported by BBSRC Anniversary Future Leader Fellowship (BB/M013170/1). J.K.F.W. was supported by MRC (MR/M007642/1) and the Royal College of Surgeons of Edinburgh support grants (SRG/14/074 & KAE WONJ4). G.C. was supported by BHF (PG/14/1/30549), MRC (MR/P016006/1), Duchenne Parent Project (Italy) and Fundació La Marató grants.

DISCLOSURES
None
REFERENCES


DOI: 10.1161/CIRCRESAHA.117.312111


43. Dora KA, Doyle MP, Duling BR. Elevation of intracellular calcium in smooth muscle causes endothelial cell generation of no in arterioles. Proc Natl Acad Sci U S A. 1997;94:6529-6534
60. Lawson D, Harrison M, Shapland C. Fibroblast transgelin and smooth muscle sm22alpha are the same protein, the expression of which is down-regulated in many cell lines. Cell Motil Cytoskeleton. 1997;38:250-257

FIGURE LEGENDS

**Figure 1.** CD146 is transiently expressed in developing aorta and regulates vascular smooth muscle maturation. **A,** Descending aorta stained for SMA, NG2 and CD146 at E8.5, E10.5, E16.5. CD146 and NG2 are expressed in VSMCs at E10.5. CD146 expression is downregulated in aortic VSMCs by E16.5, but maintained in smaller arterial branches and microvasculature (arrows). Right-hand panels illustrate delayed maturation of the abdominal aorta. At E10.5 it is not completely fused, but has CD146⁺ VSMCs. At E16.5 abdominal aorta VSMCs express CD146. **B,** In adult mouse thoracic aorta NG2 is expressed in VSMCs whereas CD146 shows only scattered expression. CD31 labels endothelium. Boxed area is magnified. Immunohistochemistry was replicated in 4 independent samples. **C,** CRISPR-Cas9 was used to knockout CD146 in 10T1/2 cells, generating cell lines C149 and C164. VSMC differentiation was induced by 48 h TGFβ1 (+T) exposure. RT-qPCR was used to characterize expression levels of Sma (Acta2), Tagln and Pcna (Proliferating cell nuclear antigen) relative to housekeeping gene Rpl19 (60S ribosomal protein L19). Biological and technical triplicate, +/- SD. Statistical significance was analyzed by Dunnett’s test by comparing untreated C149 and C164 cells to untreated WT cells and TGFβ1 treated knockout cells to corresponding TGFβ1 treated control cells. Additional data in Online Tables I-II. *** p<0.001, ** p<0.01, * p<0.05). CD146 knockout significantly increases smooth muscle contractile protein expression and reduces expression of cell proliferation marker Pcna. **D,** Immunocytochemistry illustrates enhanced expression of SMA and TAGLN in cell lines C149 and C164. Single confocal plane is shown in A and B. Scale bars: 50 μm.

**Figure 2.** Generation of mouse model to visualize VSMC differentiation. **A,** CD146-T2F transgene DNA construct. **B,** Mating scheme: TAM administration in double-transgenic CD146-T2F:NG2-CRE-ERTM line labels CD146⁺NG2⁺ VSMCs with tdTomato. Crossing the offspring to flippase reporter RCE-FRT marks CD146⁺NG2⁺ cells by tdTomato and GFP. Cells that lose the expression of CD146 downregulate tdTomato, but remain GFP⁺. **C-J,** Analysis of tdTomato expression in the CD146-T2F:NG2-CRE-ERTM line relative to native CD146 (green) and NG2 (yellow). Boxed areas magnified on the right. **C,** TAM injection at E10.5 labels CD146⁺NG2⁺ VSMCs in the dorsal aorta by E12.5. Endothelial cells (EC, arrow) express only CD146 and remain unlabeled. **D,** tdTomato expression in the femoral artery at E12.5. Limb chondrogenic cells express only NG2 and remain unlabeled. **E,** Microvasculature (endothelium is CD146⁺) in the limb lacks NG2⁺ perivascular cells and tdTomato expression at E12.5. **F-J,** TAM injection at E14.5 and analysis at E16.5. **F,** TAM administration at E14.5 labels CD146⁺NG2⁺ VSMCs in the thoracic aorta. NG2 and tdTomato expression are absent from the vein. **G,** tdTomato expression is evident in pulmonary arteries and lung pericytes (arrowhead). **H,** Abdominal aorta is marked by tdTomato, whereas vein remains unlabeled. **I,** tdTomato expression in renal arteries and glomerular perivascular cells. **J,** tdTomato⁺ perivascular cells in limb skeletal muscle at E16.5. Staining pattern was validated in 6 independent samples. All images represent single confocal planes. Scale bars: 25 μm.

**Figure 3.** Early embryonic proliferation of aortic VSMCs generates the progenitors of adult aorta VSMCs. Fate mapping analysis was performed with the triple transgenic strain. VSMCs identified by TAGLN staining. **A,** TAM administration at E10.5 leads by E15.5 to extensive VSMC labelling by tdTomato and GFP in the thoracic aorta and **B,** in the abdominal aorta. **C,** Quantification of tdTomato⁺ and GFP⁺ VSMCs at different time points in abdominal and thoracic aortae. Pulse-chase time periods indicated underneath (n=4; box plot with median indicated in boxes with lines, mean values as dots and whiskers showing highest/lowest values). **D,** TAM pulse at E14.5 leads to scarce labelling of VSMCs in the thoracic aorta at E17.5. **E,** At the same time strong marking of abdominal aorta VSMCs is visible. **F-H,** Postnatal (P6-P8) TAM administration marks few cells in thoracic and abdominal aortae with GFP at P22. **I,** Global organ level reduction of tdTomato expression can be detected in aging. tdTomato⁺ cells were FACS sorted from hind limb skeletal muscles and lung at P22 and P54 (n=3; error bars: SD; Unpaired two-tailed t-test ** p<0.0014, * p=0.022; additional statistics in Online Table III). **J-K,** Early embryonic TAM pulse (E10.5)
marks VSMC lineages that are maintained to adulthood. Endothelium stained with CD31. Single confocal plane is shown in A-B, D-G and J. Scale bars: A-B, D-G and J, 50 μm; H, K, 200 μm.

**Figure 4. Immature VSMCs are maintained at arterial branching sites.** A, At E16.5 strong CD146 staining (arrow) is evident at intersegmental (intercostal) branching site of thoracic aorta. B, TAM pulse at E14.5 labels cells with tdTomato and GFP at the intersegmental (intercostal) artery branching site of the aorta, whereas few cells are marked in the aortic wall (single channel of the boxed area magnified on the right). C, Enhanced CD146 labelling (arrow) at renal artery branch site is detectable in 3 month old mouse. D, Postnatal TAM administration (P6-P8) leads by P22 to tdTomato and GFP labelling at renal artery branching site from the abdominal aorta (boxed area magnified on the right). Branch site cells express TAGLN. E, Branch site CD146 VSMCs are defined in early embryonic development and maintained to adulthood. TAM was administered at E10.5 and renal artery branch site analyzed at P35 (boxed area magnified on the right). F-G, TAM administration in adult mice (5 doses at 3 months, analysis after 2 weeks) labels VSMCs in the SFA with GFP and tdTomato (stereo microscope image). Arrows indicate elevated tdTomato expression at branch points. H, Quantification of GFP’tdTomato+ and GFP’tdTomato– VSMCs in adult mouse SFA (n=6; +/- SD). In contrast to the descending aorta VSMCs are marked by tdTomato in the SFA. I, tdTomato is more highly expressed at SFA and descending genicular artery branch site (arrow; maximum projection of 3D confocal stack). TAM administration as before. Right-hand image demonstrates tdTomato signal intensity in a scale where white indicates strongest signal and dark blue and black lowest signal strength. J, TdTomato+ cells are visible in the walls of mesenteric artery and are enriched at its jejunal branch site (arrow; 3D reconstruction of confocal image stack). Single confocal plane is shown in A-E. Scale bars: A-B, C, D-E, I-J, 50 μm; magnified separate channels of B, D, E, 25 μm; F-G, 200 μm.

**Figure 5. Functional analysis of CD146+ VSMCs.** A, The percentage of proliferative VSMCs at E10.5, E16.5, P10 and adult (AD – 3 month) was quantified by staining for KI67 (n=4, except SFA n=7; SD is shown). AA – abdominal aorta; TA – thoracic aorta; BR – intercostal branch site; SFA – superficial femoral artery. **** p<0.0001, * p<0.05; ns – not significant; Dunnett’s test was used for comparing E10.5 to E16.5 TA and AA; unpaired two-tailed t-test was used for comparing pairs of samples at later stages; additional statistical data in Online Table IV. B, A fraction of TdTomato+ progenitor cells at renal artery branch site of the abdominal aorta at P22 are marked by KI67. C, Immature VSMCs at intercostal artery branching site show limited expression of SMMHC in comparison to the aortic wall in adult mouse. D-E, 10 μM phenylephrine (PE) causes rapid but transient rise in Ca2+ concentration in immature VSMCs at mesenteric artery branch site (n=5, SD is shown). Fluor-4 AM dye fluorescence intensity was measured before and after PE addition by using ex vivo confocal imaging. F, In vitro cell adhesion assay. Wild-type 10T1/2 or CD146 knockout cells (C149, C164) were induced to smooth muscle differentiation by 2 day exposure to 5 ng/ml TGFβ1. Cells were trypsinyzed, labelled with green fluorescent cell membrane linker and allowed to adhere to Matrigel coated surface. After 1 h the wells were washed 3 times with PBS and fluorescence intensity was quantified. G, Fluorescence spectrometry quantification of cell adhesion. Background normalized signal intensity with SD is shown (n=6). Dunnett’s test was used to calculate significance (**** p≤0.0001; **** p<0.0001, see also Online Table V). H, Aortic branching site (renal artery branching, cushion-like structures indicated by arrow) maintain high expression of pYAP1 in comparison to mature VSMCs in the aortic wall. Single confocal plane is shown in B, C, E and H. Scale bars: B-C, H, 50 μm; E, 25 μm.

**Figure 6. Distinct response of arterial VSMCs to minor and major injuries.** A, Eosin-Hematoxylin staining of healthy SFA. B, Neointima is visible (arrow) in SFA 3 weeks after wire-induced injury. C, Quantification of GFP’tdTomato+ and GFP’tdTomato– neointima cells (n=5; +/- SD; statistical data in Online Table VI). D, GFP’tdTomato+ neointima cells (arrow) surround CD31+ endothelium and E, are labelled by SMA. F, SFA super-microanastomosis by 6 sutures. G, 48 h after anastomosis transient partial thrombus forms. H, Thrombus was not evident at 2 weeks following anastomosis. I-J, Super-microanastomosis leads to replacement of GFP+ VSMCs by 2 weeks after surgery. I, Intact endothelium is
visible 2 weeks after anastomosis. J, The extent of VSMC replacement indicated with a line. Asterisk – site of injury. Bright field image is shown in the upper corner. Single confocal plane is shown in D, E and I. Scale bars: 50 μm.

Figure 7. Spatially determined response to severe arterial injury. A, Schematic of artery with distinct zones relative to the site of microanastomosis. Zone 1 lies distal from the site of anastomosis. Zone 2 encompasses the site of anastomosis. Zones 3 and 4 lie proximal from the site of anastomosis. B, Visualization of VSMC regeneration 2 weeks following super-microanastomosis. TAGLN identifies VSMCs. Triple-transgenic mice were injected for 5 days with TAM before the surgery. In zone 1 normal vascular wall is evident with GFP+tdTomato+ VSMCs. In zone 2 expanded TAGLN+ area is seen, yet these cells do not express GFP, which is only evident in microvascular pericytes. In zone 3 disorganized layers of tdTomato’GFP’ cells can be detected. Arterial wall in zone 4 is indistinguishable from healthy artery. C, SMMHC staining is evident in the arterial wall in zone 2. These cells do not arise from GFP+ VSMCs. Single confocal plane is shown. Scale bars: 50 μm.

Figure 8. Adventitial cells proliferate at site of arterial anastomosis and VSMCs distally from the site of injury. A, Following microanastomosis GFP+ VSMCs proliferate in Zone 3. Triple-transgenic 3 months old mice were injected with TAM for 5 days before anastomosis and analyzed 2 weeks later. Ki67 marks proliferative cells and SMA VSMCs. B, In zone 2, where the injury is most severe, existing VSMCs are replaced by CD44+ cells that are frequently Ki67+. C, The adventitial cells are marked by SCA1. D, Quantification of Ki67’GFP’ VSMCs in control and operated SFA (*** p=0.0044, statistical data in Online Table VII; n=4 for SFA anastomosis and n=7 for control). E, Quantification of proliferative (Ki67+’CD44+’ and SCA1+’ cells at the site of microanastomosis (n=4; +/- SD; see also Online Table VIII). F, CD44 staining is very limited in control SFA. Separate channels are shown in Online Figure VII A. G, SCA1 staining is limited to the adventitia in control artery. Separate channels are shown in Online Figure VII B. Single confocal plane is shown. H, Model for arterial VSMC differentiation dynamics in development and regeneration. In embryonic aorta CD146 is highly expressed in proliferative VSMC progenitors where it regulates the balance between contractile protein expression and proliferation. Self-renewing CD146+ immature VSMCs are maintained in cushion-like structures at postnatal arterial branch sites. Active phosphorylated YAP1 in these cells may control CD146 expression and CD146 inhibits terminal differentiation of VSMCs. Unlike the aorta smaller arteries maintain CD146 expression in adulthood. Arterial wall regeneration following injury involves multiple cell types. Where injury is most severe the resident VSMCs are replaced by adventitial cells that differentiate into new VSMCs. In case of mild intimal injury local VSMCs mount a proliferative response and contribute to neointima formation and vascular wall remodeling. Scale bars: 50 μm.
Novelty and Significance

What Is Known?

- Vascular smooth muscle cells originate from different embryonic cell types.
- Upon injury, vascular smooth muscle cells proliferate and contribute to the pathological thickening of the vascular wall.

What New Information Does This Article Contribute?

- Primitive vascular smooth muscle progenitor cells divide extensively in early embryonic development to generate long-living cell lineages that make up most of the vascular wall in the adult aorta.
- A specific immature vascular smooth muscle cell population is maintained at arterial branching sites.
- In response to minor arterial injury local smooth muscle cells switch to a proliferative phase and contribute to vascular wall thickening (hyperplasia), whereas severe surgical injury leads to smooth muscle death and recruitment of adventitial cells to the vascular wall.

Understanding when and how smooth muscle cells are replaced in blood vessel walls has important implications in cardiovascular and reconstructive surgery. Unrecognized heterogeneity in the arterial wall may influence the susceptibility of different areas of the vasculature to cardiovascular diseases, as for instance arterial branching sites are prone to atherosclerosis. We show that the origin of aortic vascular smooth muscle cells can be traced back to rapidly dividing progenitor cells that colonize the aorta shortly after its formation in the embryo. We describe a unique immature smooth muscle cell population that is maintained at arterial branching sites and reveal its molecular signature. We provide the first comparative analysis of the response of the arterial wall to minor and severe injuries. We show that resident vascular smooth muscle cells proliferate and cause arterial wall thickening in response to injury that is restricted to the luminal (intimal) side of the artery. In contrast, complete surgical transection and reconnection of the arterial wall (anastomosis) leads to the replacement of smooth muscle cells by adventitial cells and their smooth muscle differentiation. Our study offers the first complete overview of vascular smooth muscle turnover from embryonic development to arterial repair in the adult.
FIGURE 2

A

insulator  CD146 promoter  Lox-STOP-Lox  poly-A-WPRE  CD146 intron  insulator

perivascular cell

B

NG2-CRE-ERTM × CD146-T2F  →  CD146-T2F; NG2-CRE-ERTM  ×  RCE-FRT  (GFP)  →  CD146-T2F; NG2-CRE-ERTM  RCE-FRT  (GFP)

C

E10.5 - E12.5

Dorsal aorta

E

E10.5 - E12.5

Cartilage

F

E14.5 - E16.5

Thoracic aorta

G

E14.5 - E16.5

Lung

H

E14.5 - E16.5

Vein

I

E14.5 - E16.5

Kidney

J

E14.5 - E16.5

Hindlimb
Distinct Cellular Mechanisms Underlie Smooth Muscle Turnover in Vascular Development and Repair
Urmas Roostalu, Bashar Aldeiri, Alessandra Albertini, Neil E Humphreys, Maj Simonsen-Jackson, Jason K Wong and Giulio Cossu

Circ Res. published online November 22, 2017;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2017 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2017/11/21/CIRCRESAHA.117.312111
Free via Open Access

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2017/11/21/CIRCRESAHA.117.312111.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Long In Vivo Checklist

Circulation Research - Preclinical Animal Testing: A detailed checklist has been developed as a prerequisite for every publication involving preclinical studies of experimental treatments in animals. Checklist items must be clearly presented in the manuscript, and if an item is not adhered to, an explanation should be provided. If this information (checklist items and/or explanations) cannot be included in the main manuscript because of space limitations, please include it in an online supplement. If the manuscript is accepted, this checklist will be published as an online supplement. See the explanatory editorial for further information.

This study involves testing of therapeutic or diagnostic agent in animal models:
Yes

Study Design

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study.
Yes

An overall study timeline is provided.
Yes

The protocol was prospectively written.
Yes

The primary and secondary endpoints are specified.
Yes

For primary endpoints, a description is provided as to how the type I error multiplicity issue was addressed (e.g., correction for multiple comparisons was or was not used and why). (Note: correction for multiple comparisons is not necessary if the study was exploratory or hypothesis-generating in nature).
N/A

A description of the control group is provided including whether it matched the treated groups.
Yes

Inclusion and Exclusion criteria

Inclusion and exclusion criteria for enrollment into the study were defined and are reported in the manuscript.
Yes

These criteria were set a priori (before commencing the study).
Yes

Randomization

Animals were randomly assigned to the experimental groups. If random assignment was not used, adequate explanation has been provided.
Yes

Type and methods of randomization have been described.
Yes

Allocation concealment was used.
N/A

Methods used for allocation concealment have been reported.
N/A

Blinding

Blinding procedures with regard to masking of group/treatment assignment from the experimenter were used and are described. The rationale for nonblinding of the experimenter has been provided, if such was not performed.
N/A

Blinding procedures with regard to masking of group assignment during outcome assessment were used and are described.
Yes

If blinding was not performed, the rationale for nonblinding of the person(s) analyzing outcome has been provided.
N/A

Sample size and power calculations

Formal sample size and power calculations were conducted before commencing the study based on a priori determined outcome(s) and treatment effect(s), and the data are reported.
N/A

If formal sample size and power calculation was not conducted, a rationale has been provided.
Yes

Data Reporting

DOI: 10.1161/CIRCRESAHA.117.312111
Baseline characteristics (species, sex, age, strain, chow, bedding, and source) of animals are reported.

The number of animals in each group that were randomized, tested, and excluded and that died is reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided for all experimental groups.

Baseline data on assessed outcome(s) for all experimental groups are reported.

Details on important adverse events and death of animals during the course of the experiment are reported for all experimental groups.

Numeric data on outcomes are provided in the text or in a tabular format in the main article or as supplementary tables, in addition to the figures.

To the extent possible, data are reported as dot plots as opposed to bar graphs, especially for small sample size groups.

In the online Supplemental Material, methods are described in sufficient detail to enable full replication of the study.

Statistical methods

The statistical methods used for each data set are described.

For each statistical test, the effect size with its standard error and $P$ value is presented. Authors are encouraged to provide 95% confidence intervals for important comparisons.

Central tendency and dispersion of the data are examined, particularly for small data sets.

Nonparametric tests are used for data that are not normally distributed.

Two-sided $P$ values are used.

In studies that are not exploratory or hypothesis-generating in nature, corrections for multiple hypotheses testing and multiple comparisons are performed.

In “negative” studies or null findings, the probability of a type II error is reported.

Experimental details, ethics, and funding statements

Details on experimentation including formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring are described.

Both male and female animals have been used. If not, the reason/justification is provided.

Statements on approval by ethics boards and ethical conduct of studies are provided.

Statements on funding and conflicts of interests are provided.

Date completed: 11/20/2017 11:12:59
User pid: 409159

DOI: 10.1161/CIRCRESAHA.117.312111
SUPPLEMENTAL MATERIALS AND METHODS

Cell culture

Authenticated 10T1/2 cells originate from European Collection of Authenticated Cell Cultures, Public Health England (Porton Down, UK) and were cultured in MEM (Gibco, ThermoFisher, Waltham, MA, USA), supplemented with 1% L-Glutamine, 10% foetal bovine serum (FBS), 1% penicillin-streptomycin, 1x MEM Non-Essential Amino Acids Solution. Smooth muscle differentiation was carried out according to published protocol. The cells were serum starved for 24 h before adding medium containing 5 ng/ml recombinant TGFβ1 for 2 days (Cat. No. 7666-MB-005; R&D Systems, Minneapolis, USA). For RNA extraction cells were cultured in 48 well plates and for immunocytochemistry in glass-bottom 24-well Sensoplates (Cat. No. 662892; Greiner Bio-One, Stonehouse, UK).

We have described the generation of CD146 knockout cell lines and their mutations previously. Both C149 and C164 cell lines carry premature STOP codons in the second exon of CD146, encoding the first N-terminal immunoglobulin domain.

Cell adhesion assay

Cells were induced to smooth muscle differentiation as described above, trypsinized and washed once with growth medium containing 10% FBS and twice with medium without serum (each step followed by 5 minute centrifugation at ambient temperature at 500 g). Cells were labelled with PKH67 green fluorescent cell linker (Sigma Aldrich, St. Louis, MO, USA) according to standard protocol after which the cells were washed three times with growth medium to eliminate unbound linkers. Equal amount of cells (30,000) were seeded in 96-well NUNC plate wells (ThermoFisher, Waltham, MA, USA) in 100 μl MEM (Gibco/Thermo Fisher), supplemented with 1% L-Glutamine, 10% FBS, 1% penicillin-streptomycin, 1x MEM Non-Essential Amino Acids Solution. Plates had previously been coated with phenol red free Matrigel matrix (Cornig, New York, USA) according to manufacturer’s protocol to achieve thin uniform layer. After 1 h the plates with cells were removed from 37°C incubator, imaged using inverted microscope, and rapidly blotted on paper to remove medium. The wells were washed three times with 37°C PBS and fluorescence intensity was measured with Bio-Rad iMark plate reader (Bio-Rad Laboratories, Hercules, CA, USA). Each cell line was measured in 6 wells, the measurements were normalized to background fluorescence.

Transgenic mouse model generation

The DNA construct for transgene was compiled of several fragments. Ai9 plasmid was a gift from Hongkui Zeng (Allen Institute for Brain Science, Seattle, WA, USA, Addgene plasmid # 22799). We replaced tdTomato STOP codon in Ai9 by multicloning site, where we inserted P2A-NLS-Flpo (self-cleaving peptide linked to nuclear localized flippase). pFLPo was a gift from Philippe Soriano (Icahn School of Medicine at Mount Sinai, NY, USA, Addgene plasmid # 13792). We used Ai9 as a template and mutated LoxP sites to Lox2272 sites in order to avoid possible recombination in the triple transgenic mice, as flippase reporter strain RCE-FRT has LoxP site that was used in its generation. We cloned together fragment containing Lox2272-STOP-Lox2272-Kozak-tdTomato-P2A-NLS-Flpo-WPRE-polyA. We next synthesized 12292 bp long DNA fragment corresponding to the upstream genomic region and the first intron of mouse Mcam (CD146). It included chicken β-globin HS4 insulator on the 3’ side. The synthesized DNA included unique restriction enzyme sites that allowed us to replace the first exon of Mcam by the transgene coding construct. The 12 kb long sequence was flanked by restriction enzyme sites that permitted its linearization before pronuclear injection. Thermo Scientific Phusion High-Fidelity


DNA Polymerase (Cat. No. F530L) was used in all the cloning steps, combined with Agilent SURE2 competent cells (Cat. No. 200152; Agilent Technologies Inc., Santa Clara, CA, USA). Qiagen Large-Construct Kit (Cat. No. 12462; Qiagen Gmbh, Hilden, Germany) was used for DNA isolation. Linearized DNA was purified using Qiagen Qiaex II beads (Cat. No. 20021) followed by ethanol precipitation and was eventually resuspended in Millipore EmbryoMax Injection Buffer (Merck Millipore, Billerica, MA, USA). The pronuclear injection (Harlan B6D2F1 (BDF1) wild-type mice) led to 6 founder mice that were back-crossed to C57BL/6 wild-type mice to assess germline penetrance and then to NG2-CRE-ERTM line to study fluorescence specificity and signal strength. Although all founders transmitted the transgene to offspring we detected strongest fluorescence signal in one line that was used for experiments here. The transgene expression did not cause any harmful side effect. All mice were housed in individually ventilated cages (12 h light-dark cycle) and fed standard rodent diet. Sample size for surgical procedures was estimated based on previously found variability. Both male and female mice were used for all experiments and randomly assigned to experimental and control groups. No obvious differences in aortic VSMCs were detected between sexes.

**Ex-vivo calcium imaging**

3 month old adult mice were sacrificed and the descending aorta was rapidly removed from the abdominal cavity. The aorta was cleaned outside from adipose tissue and cut into smaller segments. Calcium imaging was carried out as previously described, with some modifications. The aorta was stained with Fluo-4 AM dye (Thermo Fisher Cat. No. F14217) in Thermo Fisher Live Cell Imaging Solution (A14291DJ), supplemented with Pluronic F-127 (Thermo Fisher Cat. No. P6866) (7 μl 1 mM Fluo-4 AM, 450 μl imaging solution and 40 μl 10% water-based Pluronic F-127 per aorta). The aorta was incubated with the dye for 1 h at ambient temperature on a horizontal shaker in dark. Following the incubation period the aorta was washed 4 times in imaging solution, cut longitudinally in half and embedded with luminal side up in 1% low melting point agarose (in imaging solution). Phenylephrine hydrochloride (Sigma Aldrich, Cat. No. P6126) was dissolved in imaging solution. Leica SP8 upright confocal microscope with 25x water dip-in objective was used for imaging at 1.48 frame per second speed. Imaging was carried out in Thermo Fisher Live Cell Imaging Solution (containing 1.8 mM CaCl2). Phenylephrine was added to 10 μM final concentration. Fluorescence intensity was quantified in ImageJ for each intimal cell at the aortic branch site (Multi Measure tool for all cells and all time points).

**Immunostaining**

Samples were fixed in 4% paraformaldehyde (PFA), equilibrated in 15 and 30% Sucrose/PBS, embedded in OCT and cut in cryostat (Leica 3050S) to 7 μm thick sections. Cryosections were washed 3 times in PBS-Tween (0.1%) (PTW), blocked for 1 h in 1% bovine serum albumin (BSA) in PTW and 3 h in 10% donkey serum in PTW. Primary antibodies were incubated overnight at 4°C in 10% donkey serum. On second day the samples were washed 3 times 5 minutes and 3 times 30 minutes in PTW, incubated for 30 minutes in 1% BSA in PTW and for 2 h with secondary antibodies in PTW (ambient temperature, humidified chamber). Following the removal of the secondary antibody the samples were washed 3 times 15 minutes in PTW, 2 times 15 minutes in PBS, rinsed in distilled water and mounted using Vectashield mounting medium (Cat. No. H1000; Vector Laboratories, Burlingame, CA, USA). For staining cells for FACS, the dissociated cells were incubated in PBS and 10% donkey serum on ice for 30 minutes, spun down (700 g, 7 minutes), followed by a 30 minute incubation with primary antibodies diluted in the same buffer. After antibody incubation the cells were washed 3 times in PBS (10% serum). FACS gating was set on non-fluorescent organ samples and unstained fluorescent samples.
For immunocytochemistry cells were washed with PBS and fixed in 4% PFA for 20 minutes. The cells were washed 3 times 15 minutes in PTW and blocked for 1 h in 10% donkey serum in PTW (ambient temperature). Primary antibodies were added overnight in blocking buffer (4°C). On second day (all steps at ambient temperature) the cells were washed 4 times 20 minutes in PTW and blocked for 30 minutes in 1% BSA in PTW. Secondary antibody was added for 1 h after which the cells were washed for 3 times 15 minutes in PTW and kept in PBS for imaging.

**FACS**

Tissue was diced in Krebs-Ringer-Hepes (KRH), containing 2.5 mM glucose, 2% FBS, 2mg/ml type II Collagenase (Cat. No. LS004176; Worthington Biochemical Corp., Lakewood, NJ, USA) or 1 mg/ml collagenase XI (Sigma Aldrich Cat. No. C7657) for pancreas. Samples were lyzed in shaking water-bath at 37°C until homogenous suspension was reached (40-80 min). Cells were diluted in 5-times volume of ice-cold KRH (2% FBS), passed through 40 μm strainer, centrifuged (700 g, 7 minutes), resuspended in KRH (2% FBS), passed through 40 μm strainer and sorted using BD Bioscience FACSria.

**Antibodies**

The following antibodies were used at these indicated concentrations: Rabbit anti-RFP (Rockland 31896; 1:100), Chicken anti-GFP (Abcam Ab13970; 1:100), Rabbit anti-NG2 (Millipore AB5320; 1:100), Rat anti-NG2 (R&D MAB6689-SP; 1:100), Rat anti-CD146 (R&D MAB7718; 1:100), Sheep anti-CD146 (R&D AF6106; 1:500), Rat anti-CD31/PECAM (Dev. Studies Hybridoma Bank; 1:3), Rabbit anti-PDGFRB (Cell signaling 3169; 1:100), Mouse anti-SMA (Sigma A2547; 1:300), Goat anti-SMA (Abcam ab21027; 1:200), Rabbit anti-TAGLN (Abcam ab14106; 1:100), Rabbit anti-SMMHC (Abcam ab53219; 1:100), Rabbit anti-Ki67 (Abcam ab15580; 1:200), Rabbit anti-pYAP1 (Cell Signaling 13008T; 1:100), Rat anti-CD34 (Abcam ab8158; 1:50), Goat anti-SCA1 (R&D AF 1226; 1:30), Rat anti-CD44 (BD 563608, 1:200). Diverse Alexa-Fluor or DyLight conjugated secondary antibodies from Thermo Fisher Scientific were used at 1:500 dilution.

**RNA isolation and RT-qPCR**

RNA was extracted using TRIzol reagent (Cat. No. 15596018; Thermo Fisher Scientific) according to manufacturer’s protocol and resuspended in 20 μl water. RNA was quantified with Nanodrop 2000 and equalized to same concentration before cDNA synthesis. Reverse-transcription was performed using random hexamer primers and RevertAid First Strand cDNA Synthesis Kit (Cat. No. K1622; Thermo Fisher Scientific). RT-qPCR was carried out using FastStart Essential DNA Master Mix (Cat. No. 06402712001; Roche diagnostics GmbH, Mannheim, Germany) on a Roche LightCycler 96 system. RT-qPCR was performed in technical and biological triplicate for each sample, using standard dilution series on every plate. Roche LightCycler software version 1.5 was used for quantification. Primer sequences were acquired from Harvard PrimerBank (Spandidos et al., 2008; Spandidos et al., 2010; Wang and Seed, 2003): Acta2 (ID 6671507a1); Pena (ID 7242171a1); Rpl19 (ID 6677773a1); Tagln (ID 675514a1).

FActa2 GTCCCACACATCAGGGAGTAA, RActa2 TCGGATACTTCAGCGTCAGGA
FPena TTTGAGGCACGCTCTGATCC, RPena GGAGACGTCAGACGACCATCAT
FRPl19 ATGAGTAGCTACCCGCTAGCA, RRPl19 GCTATTGGCCCATTTCTGTC
FTagln CAACAAGGGTCCATCTACGG, RTagln ATCTGGGCGGCTACATCA
SUPPLEMENTAL REFERENCES


SUPPLEMENTAL MATERIAL

ONLINE FIGURE LEGENDS

Online Figure I. CD146 and NG2 expression dynamics in vascular smooth muscle development and the role of CD146 in cell differentiation. A, Descending aorta stained for CD31 (endothelium), NG2 and CD146 at E8.5, E10.5 and E16.5. Boxed areas are magnified. CD146 is expressed at E8.5 in the dorsal aorta endothelial cells. NG2 and CD146 expressing VSMCs appear at E10.5. Aortic smooth muscle cells maintain NG2 expression at E16.5 but downregulate CD146. CD146 expression is maintained in the endothelium (E), in microvasculature and smaller branches of the arteries (arrows). B, 10T1/2 cells upregulate SMMHC expression after 48 h exposure to TGFβ1. Stronger staining is evident in CD146 knockout cell lines C149 and C164. C, Staining for Ki67 reveals more limited cell proliferation in C149 and C164 cell lines in comparison to wild type 10T1/2 cells. Scale bars: 50 μm.

Online Figure II. Characterization of CD146/Mcam genomic region and CD146-T2F:NG2-CRE-ERTM transgenic model. A, Epigenetic modifications from ENCODE database for mouse CD146/Mcam genomic region. Profiles are shown for 8 week old mouse cerebellum, heart, kidney, small intestine, lung and embryonic E14.5 limb. The DNA region, synthesized for CD146-T2F transgene, is shown underneath in blue. Histone modifications are indicated on the right together with CTCF binding motif. B, TAM administration at E14.5 leads by E17.5 to extensive labelling of CD146^NG2^ mural cells by tdTomato (red) in double-transgenic CD146-T2F:NG2-CRE-ERTM mouse lung. Staining for native CD146 is shown in green, for NG2 in yellow and nuclei (Hoechst) in blue. C, At E12.5 tdTomato^+^ VSMCs in the aorta express PDGFRβ (TAM injection at E10.5). D, Lymphatic vasculature (LYVE1, Lymphatic vessel endothelial hyaluronan receptor 1) is not labelled in the transgenic model (TAM injection at E14.5, analysis at E16.5). E, Determination of potential Lox-STOP-Lox cassette leakiness. CRE independent read-through of the STOP cassette can lead to endothelial labelling in the triple-transgenic strain since CD146 is highly expressed in endothelial cells. In the skeletal muscle we detected less than 4% of GFP labelled endothelial cells, whereas in the pancreas and lung only scarce GFP^+^ endothelial cells were evident (n=3; error bars: SD). Juv: TAM injection P6-P8, analysis at P22. Ad: 5 TAM injection after P35, analysis at P54. F, FACS plots illustrating CD31^+^GFP^+^ cells in skeletal muscle (Gastrocnemius), pancreas and lung at P22. There is only limited leakiness of the STOP cassette and endothelial expression of GFP. Scale bars: B, 200 μm; C-D, 25 μm.

Online Figure III. Triple-transgenic model demonstrates dynamic reduction of CD146 expression in arterial smooth muscle maturation. A-D, VSMCs are visualized by SMA staining. A, Transgene expression was triggered at E14.5 and analyzed at E17.5. Only scattered tdTomato^GFP^SMA^−^ cells are evident in the thoracic aorta. B, At the same time tdTomato^GFP^SMA^−^ cells are more frequent in the abdominal aorta (quantified in Figure 3C). C, TAM administration in juvenile mice (P6-P8) labels only rare cells with GFP in the abdominal aortic wall by P22. D, TAM administration from P6 to P8 leads to efficient marking of arteriolar and microvascular mural cells in the kidney at P22. E, Very few cells are marked in the thoracic aorta when TAM was administered in adulthood (5 consecutive days) and analyzed 2 weeks after the last injection. TAGLN was used to identify aortic VSMCs. F, FACS plots illustrate decrease in tdTomato expression in adult hind limb skeletal muscles (see quantification in Figure 3I). G, NG2-CRE-ERTM:Rosa26-tdTomato mouse strain has very efficient VSMC labelling in the adult thoracic aorta. TAM was injected for 5 consecutive days starting at P35 and analyzed 2 weeks after the last injection. Single confocal plane is shown in all images. Scale bars: 50 μm.

Online Figure IV. Additional characterization of arterial branching site cells. A, TAM administration in juvenile growth period (P6-P8) leads to tdTomato and GFP labelling of progenitor cells at abdominal aorta inferior mesenteric artery branch site at P22. The cells form cushion-like structures close to CD31^+^ endothelium and vascular lumen. B, Second transgenic mouse line (BCD-146T2F) was
generated by independent pronuclear injection. This strain was crossed to NG2-CRE-ERTM line and thereafter to flippase reporter strain RCE-FRT. Postnatal TAM administration in the triple transgenic mice from P6 to P8 labelled progenitor cells at renal artery branch sites at P22 in tdTomato, whereas no red fluorescence was evident in the remaining aortic wall. The independent transgenic line confirms the existence of CD146 expressing progenitor cells at aortic branch sites. Single confocal plane is shown in all images. Scale bars: 50 μm.

Online Figure V. Characterization of VSMC turnover and the role of CD146 in VSMC adhesion. A, Descending aorta from E10.5, E16.5, P10 and 3 month old adult mice was stained for KI67 and SMA to identify proliferative VSMCs. Femoral artery from adult mouse is shown below. Only rare KI67 marked VSMCs can be seen in adult mouse thoracic aorta and femoral artery. B, Staining for activated caspase 3 (CASP3) did not reveal apoptotic VSMCs in the thoracic aorta. Note CASP3 positive cells in the surrounding tissue (boxed area is magnified). C, Cell adhesion assay was used to compare WT cells and CD146 knockout cells (C149, C164). Visualization of green fluorescent cells adhered to Matrigel before and after three PBS washes (details in Figure 5F-G). Scale bars: A-B, 50 μm; C, 200 μm.

Online Figure VI. Characterization of superficial femoral artery super-microanastomosis model. A-B, Artery was exposed, separated from the vein and nerve, clamped around the site of vessel division and repaired by super-microanastomosis (vessel repair <0.5mm Ø). C, Repair was performed in adult triple-transgenic mice, injected for 5 consecutive days with TAM before the surgery. Two weeks after super-microanastomosis pre-existing GFP+ VSMCs have been replaced. GFP expression is still maintained in VSMCs (arrow) outside the regenerative zone. In the regenerating area GFP remains visible in pericytes of the microvasculature (arrowhead). Dotted line indicates the location of the artery. Confocal maximum intensity projection of the whole 3-dimensional stack is demonstrated. D, TAGLN (SM22)-CRE mouse was crossed to Rosa26-tdTomato reporter line enabling robust labelling of VSMCs (stained for SMA, arrow), but also fibroblasts surrounding the artery. E, 48 h after SFA super-microanastomosis the existing SMA and TAGLN-CRE:tdTomato marked VSMCs (arrow) have been lost. Scale bars: F, 1mm; C, 200 μm; D-E, 50 μm.

Online Figure VII. Adventitial cells respond to severe arterial injury and microanastomosis. In all images triple-transgenic mice were used. TAM was administered for 5 consecutive days and tissue was analyzed 2-3 weeks after the final injection. A, In control (unoperated) artery very limited CD44 expression is evident. Separate channels corresponding to Fig. 8F. B, In control artery SCA1 staining is limited to the adventitial layer (asterisk) and is absent from the tunica media (arrow). Separate channels corresponding to Fig. 8G. C, Similarly to SCA1, CD34 expression marks adventitial layer (asterisk) in control artery. D, 2 weeks following microanastomosis CD34+ marked cells have penetrated the tunica media (arrow), replacing GFP+ VSMCs. E, Three weeks after SFA wire induced injury SCA1+ cells have not infiltrated tunica media (arrow). F, limited staining of CD44 is evident three weeks after SFA wire induced injury. Scale bars: 50 μm.
Online Figure I

A

CD31/CD146/NG2/Hoechst

E8.5

Thoracic aorta

E10.5

Thoracic aorta

E16.5

B

WT C149 C164

C

WT C149 C164

SMHMC

+ TGFβ1
Online Figure IV

GFP/tdTomato/CD31/Hoechst

A
Pulse-chase: P6 to P22

B
Online Figure V

**A**

- E10.5: Dorsal aorta
- E16.5: Thoracic aorta
- P10: Thoracic aorta
- 3 month: 3 month
- 3 month: Femoral artery

**B**

- SMA/Ki67/Hoechst
- SMA/CASP3/CD31/Hoechst

**C**

- After adhesion
- After washes

WT

C149

C164
**ONLINE TABLES**

**Online Table I.** Upper and lower confidence intervals for relative normalized gene expression values (corresponding to Figure 1C).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sma</td>
<td>0.35</td>
<td>0.46</td>
</tr>
<tr>
<td>Tagln</td>
<td>1.13</td>
<td>0.90</td>
</tr>
<tr>
<td>Pcna</td>
<td>1.13</td>
<td>0.90</td>
</tr>
</tbody>
</table>

**Online Table II.** Statistical significance was analysed by Dunnett’s test and the effect size as well as p-values are shown for comparison of untreated CD146 knockout cells (C149, C164) to untreated wildtype (WT) cells and for TGFβ1 treated CD146 knockout cell lines in comparison to corresponding TGFβ1 treated WT cells (corresponding to Figure 1C).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Gene</th>
<th>Mean diff.</th>
<th>95% CI of diff.</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Sma</td>
<td>-0.443</td>
<td>-0.7 to -0.187</td>
<td>0.0047</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Sma</td>
<td>-0.703</td>
<td>-1.101 to -0.306</td>
<td>0.0041</td>
</tr>
<tr>
<td>Untreated</td>
<td>Tagln</td>
<td>-0.67</td>
<td>-0.988 to -0.352</td>
<td>0.0017</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Tagln</td>
<td>-1.126</td>
<td>-1.443 to -0.808</td>
<td>0.0001</td>
</tr>
<tr>
<td>Untreated</td>
<td>Pcna</td>
<td>0.753</td>
<td>0.460 to 1.046</td>
<td>0.0006</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Pcna</td>
<td>0.377</td>
<td>0.083 to 0.669</td>
<td>0.0183</td>
</tr>
</tbody>
</table>
Online Table III. Loss of tdTomato labelling in aging was analysed by comparing juvenile (P22) skeletal muscle and lung to adult mouse (P54) corresponding tissues by FACS. Statistical significance was analysed by unpaired two-tailed t-test. Corresponding to Figure 3I.

<table>
<thead>
<tr>
<th></th>
<th>P22 muscle</th>
<th>P54 muscle</th>
<th>P22 lung</th>
<th>P54 muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.832</td>
<td>0.120</td>
<td>0.042</td>
<td>0.014</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>0.135</td>
<td>0.08</td>
<td>0.008</td>
<td>0.012</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>0.497</td>
<td>-0.079</td>
<td>0.024</td>
<td>-0.015</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>1.167</td>
<td>0.319</td>
<td>0.061</td>
<td>0.042</td>
</tr>
<tr>
<td>Difference between means</td>
<td>-0.712 ± 0.091</td>
<td>-0.029 ± 0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% CI of diff.</td>
<td>-0.963 to -0.461</td>
<td>-0.051 to -0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>0.0014</td>
<td>0.022</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Online Table IV. VSMC proliferation analysis. % of KI67⁺ VSMCs was quantified in the dorsal aortae of E10.5 mouse embryos and in the abdominal (AA) and thoracic (TA) aortae of E16.5, P10 and adult (AD, 3 months) mice, and in the branch sites of intercostal arteries from the aorta (BR) as well as in the superficial femoral arteries (SFA) of adult mice. Corresponding to Figure 5A.

<table>
<thead>
<tr>
<th></th>
<th>E10</th>
<th>E16 AA</th>
<th>E16 TA</th>
<th>P10 AA</th>
<th>P10 TA</th>
<th>AD AA</th>
<th>AD TA</th>
<th>AD BR</th>
<th>AD SFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>92.98</td>
<td>30.22</td>
<td>25.54</td>
<td>17.97</td>
<td>14.78</td>
<td>0.359</td>
<td>0.77</td>
<td>3.706</td>
<td>0.653</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>2.41</td>
<td>1.305</td>
<td>2.197</td>
<td>2.071</td>
<td>4.158</td>
<td>0.41</td>
<td>0.06</td>
<td>3.026</td>
<td>0.950</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>89.15</td>
<td>28.15</td>
<td>22.04</td>
<td>14.68</td>
<td>8.164</td>
<td>-0.29</td>
<td>0.675</td>
<td>-1.109</td>
<td>-0.225</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>96.82</td>
<td>32.3</td>
<td>29.03</td>
<td>21.27</td>
<td>21.4</td>
<td>1.01</td>
<td>0.865</td>
<td>8.522</td>
<td>1.532</td>
</tr>
<tr>
<td>Dunnett’s test</td>
<td>E10 vs. E16 AA and TA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difference between means (vs. E10)</td>
<td>62.76 ± 1.512</td>
<td>67.45 ± 1.512</td>
<td>58.49 to 63.18 to</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% CI of diff.</td>
<td>67.03</td>
<td>71.72</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two-tailed t-test</td>
<td>E16 AA vs TA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difference between means</td>
<td>-4.687 ± 1.278</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% CI of diff.</td>
<td>-7.814 to -1.561</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>0.0105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Online Table V. Statistical analysis of cell adhesion of CRISPR-Cas9 edited cell lines. Cell adhesion was studied by fluorescence spectrometry and Dunnett’s test was used to analyse significance between WT cells and mutant cells (C149 and C164). Corresponding to Figure 5G.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>C149</th>
<th>C164</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.399</td>
<td>0.107</td>
<td>0.209</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>0.055</td>
<td>0.032</td>
<td>0.08</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>0.341</td>
<td>0.072</td>
<td>0.124</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>0.456</td>
<td>0.141</td>
<td>0.293</td>
</tr>
<tr>
<td>Difference between means (vs. WT)</td>
<td>0.292</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>95% CI of diff. (WT, C149)</td>
<td>0.209 to 0.375</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% CI of diff. (WT, C164)</td>
<td>0.107 to 0.273</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value (in comparison to WT)</td>
<td>0.0001</td>
<td>0.000107</td>
<td></td>
</tr>
</tbody>
</table>

Online Table VI. Statistical analysis of GFP and tdTomato labelling in neointima cells 3 weeks after wire induced injury (mean % of total neointima cells; corresponding to Figure 6C).

<table>
<thead>
<tr>
<th></th>
<th>GFP’tdT+</th>
<th>GFP’tdT-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>51.75</td>
<td>4.775</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>4.423</td>
<td>2.382</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>1.978</td>
<td>1.065</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>46.25</td>
<td>1.818</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>57.24</td>
<td>7.733</td>
</tr>
</tbody>
</table>

Online Table VII. SFA anastomosis causes VSMC proliferation. Statistical significance in comparison to unoperated SFA was analysed by unpaired two-tailed t-test (mean indicates % from total VSMCs; corresponding to Figure 8D).

<table>
<thead>
<tr>
<th></th>
<th>Unoperated control SFA</th>
<th>Anastomosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.653</td>
<td>22.56</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>0.950</td>
<td>3.987</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>-0.225</td>
<td>16.21</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>1.532</td>
<td>28.9</td>
</tr>
<tr>
<td>Difference between means</td>
<td>21.91 ± 1.523</td>
<td></td>
</tr>
<tr>
<td>95% CI of diff.</td>
<td>18.46 to 25.35</td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>0.0044</td>
<td></td>
</tr>
</tbody>
</table>
Online Table VIII. SFA anastomosis causes adventitial cell proliferation. Corresponding to Figure 8E.

<table>
<thead>
<tr>
<th></th>
<th>% of Ki67⁺SCAI⁺ cells</th>
<th>% of Ki67⁺CD44⁺ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>13.29</td>
<td>26.16</td>
</tr>
<tr>
<td><strong>Std. Deviation</strong></td>
<td>1.932</td>
<td>10.67</td>
</tr>
<tr>
<td><strong>Lower 95% CI of mean</strong></td>
<td>10.21</td>
<td>9.192</td>
</tr>
<tr>
<td><strong>Upper 95% CI of mean</strong></td>
<td>16.36</td>
<td>43.14</td>
</tr>
</tbody>
</table>