PROMOTING ELASTOGENESIS IN ARTERIAL AND VENOUS VASCULAR SMOOTH MUSCLE CELLS AND FIBROBLASTS FOR VASCULAR TISSUE ENGINEERING

T.C. Rothuizen[1], R. Kemp[1], J.M.G.J. Duijs[1], H.C. de Boer[1], R. Bijkerk[1], E.P. van der Veer[1], L. Moroni[2], A.J. van Zonneveld[1], A.S. Weiss[3], T.J. Rabelink[1], J.I. Rotmans[1]

[1] Department of Internal Medicine, Section Nephrology and Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, The Netherlands
[2] MERLN Institute for Technology Inspired Regenerative Medicine, Complex Tissue Regeneration, Maastricht University, The Netherlands
[3] School of Molecular Bioscience and Charles Perkins Centre and Bosch Institute, The University of Sydney, Australia.

PLoS One. 2015 Nov 20;10(11)

ABSTRACT

Background

Elastin remains a missing link in vascular tissue engineering and has important structural and regulatory functions. Adult arterial and venous vascular smooth muscle cells (VSMCs) and fibroblasts are mainly used for vascular tissue engineering approaches and are key elastogenic cells. Many elastin-inductive compounds have been reported, but their relative efficiency in promoting elastogenesis by these four cell types has never been assessed. In addition to positive elastin regulators, microRNA-29a is a potent negative post-transcriptional regulator of elastogenesis. We explored whether stimulating positive regulation or blocking negative regulation of elastin synthesis is more effective in elastin production.

Methods

We compared the elasto-inductive capacity of three known mRNA upregulating agents IGF-1, TGF-β1 and minoxidil, and a miR-29a-inhibitor in adult venous and arterial fibroblasts and VSMCs. The effects on elastin, lysyl oxidase and fibrillin-1 mRNA expression and tropoelastin protein was evaluated.

Results

IGF-1 and minoxidil exerted little effect on tropoelastin mRNA and protein levels for all cell types, while TGF-β1 predominantly enhanced tropoelastin mRNA expression but lacked an effect at the protein level. In contrast, microRNA29a-inhibition resulted in the upregulation of elastin mRNA in all cell types which was most pronounced in venous VSMCs. This inhibition also enhanced lysyl oxidase and fibrillin-1 mRNA and resulted in a dose dependent increase in tropoelastin protein expression in venous VSMCs.

Conclusion

The elastogenic potential of microRNA29a-inhibition in vascular cells is superior to that of IGF-1, TGF-β1 and minoxidil. On this basis, miR-29a-inhibitors present an attractive target to enhance elastin synthesis in tissue engineered blood vessels.

INTRODUCTION

Tissue engineered blood vessels (TEBVs) hold great promise as vascular substitutes. Despite its potential, the formation of functional elastic fibers remains one of the missing links in TEBVs(1). Elastin is secreted into the extracellular space as a soluble tropoelastin monomer, which is oxidized by lysyl oxidase and subsequently deposited as crosslinked protein onto fibrillin-rich microfibrils to form mature elastin fibers(2). Elastogenesis mainly occurs in a late fetal- and early neonatal period, and is notably absent in mature tissues(3;4). As such, generating elastin-rich TEBVs has proven to be an extreme challenge(1).

In the arterial medial wall, the elastic fibers are organized in concentric rings of elastic lamellae, which are responsible for the vessel's resilience and compliance(5;6). Next to the mechanical properties, the elastic lamellae form a structural barrier, thus preventing migration of adventitial fibroblasts and medial vascular smooth muscle cells (VSMCs) to the intima. In addition, elastin signaling plays a critical role in VSMC activity by regulating proliferation and migration(7). Indeed, the complete absence of elastin has been shown to be lethal in elastin knockout mice due to severe intimal hyperplasia (8). Therefore, the incorporation of elastic fibers may be pivotal to generate TEBVs with adequate vascular function.

In vascular tissue engineering, adult arterial and venous VSMCs and fibroblasts are frequently used as the main cell sources(9-13). Although tropoelastin can be synthesized by several cell types(14;15), arterial and venous VSMCs and fibroblasts are amongst the main elastogenic cells(16). Many elastogenic compounds have been reported, but no comparison has been made in their efficiency to induce tropoelastin in these four cell types. This issue is important, as there are marked differences in phenotype, function and response to physical and chemical stimuli between arterial and venous cells, and between fibroblasts and VSMCs(17-22). In addition to stimulatory compounds, microRNA-29a (miR-29a) has recently been described in post-translational downregulation of tropoelastin synthesis(23;24). Although both tropoelastin mRNA inductive and miR-29 inhibiting agents have shown promising results(24;25), we wanted to determine which method is the most efficacious in increasing elastogenesis by vascular cells.

Here, we studied the difference in elastogenic capacity of three potent mRNA upregulating agents IGF-1, TGF-β1 and minoxidil sulfate, and a miR-29a inhibitor in venous and arterial fibroblasts and VSMCs.

MATERIALS & METHODS

Cell isolation

Medial VSMCs and adventitial fibroblasts were isolated from both a carotid artery and a jugular vein from female Dutch Landrace adult pigs. Thus, in total four cell types were isolated. For fibroblasts, the adventitia was stripped from the medial layer, minced and incubated with collagenase A (Sigma, Germany) for 3h. After centrifuging the suspension at 1.500 rpm for 5 min, the remaining cell pellet was resuspended in fibroblast medium and plated. After isolation of the fibroblasts from the adventitia, the remainder of the blood

vessel was used to isolate the VSMCs. For this, the vessels were dissected, the endothelial layer was gently scraped of using tweezers and the medial layer was cut into small pieces. These pieces were pinned with 25G needles to the bottom of a Petri dish with the luminal side facing downwards for direct outgrowth of VSMCs.

Cells were characterized with immunocytochemistry to confirm successful isolation. Fibroblast phenotype was defined as vimentin(+), α-SMA(-)cells. VSMCs were defined as vimentin(+), α-SMA(+) cells. Primary antibodies against vimentin (Immunologic, The Netherlands, 1:1000) and α-SMA (Dako, The Netherlands, 1:1000) and secondary antibodies labelled with Alexa568 (LifeTech, The Netherlands) were used and cells were counterstained with Hoechst. In each case, a negative isotype control was performed. Images were captured using an inverted fluorescence microscope (Leica DM1-600, Germany).

Cell culture

For arterial and venous VSMCs, Medium 199 (PAA/GE Healtcare, Austria) supplemented with heat inactivated 10% fetal calf serum, 1% penicillin/streptomycin (PAA/GE Healthcare, Austria), 0.25µg/mL a*mphotericin B* (Gibco, LifeTech, The Netherlands), 10mM Hepes (PAA/GE Healthcare, Austria) and 4mM L-glutamine (PAA/GE Healthcare, Austria) was used. For arterial and venous fibroblasts, DMEM (PAA/GE Healthcare, Austria) supplemented with heat inactivated 10% fetal calf serum, 1% penicillin/streptomycin (PAA/GE Healthcare, Austria), 0.25µg/mL a*mphotericin B* (Gibco, LifeTech, The Netherlands) and 1mM sodium pyruvate (PAA/GE Healthcare, Austria) and 2mM L-glutamine (PAA/GE Healthcare, Austria) was used. All cells were incubated at 37°C and 5% CO_2. For experiments, cells in passage 4-5 were used.

For mRNA isolation, cells were cultured in 12-well plates at a density of 120.000 cells/well (80% confluency) for VSMCs and 150.000 cells/well (80% confluency) for fibroblasts. For protein isolation, cells were cultured in 6-wells plates at a density of 300.000 cells/well (80% confluency) for VSMCs and 350.000 cells/well (80% confluency) for fibroblasts.

Experimental conditions

For each of the 4 cell types (arterial and venous VSMCs and fibroblasts), 4 different elastogenic stimuli were evaluated: TGF-β1, minoxidil sulfate, IGF-1 and a microRNA-29a inhibitor (LNA-29a). TGF-β1 (LifeTech, Germany) was dissolved in acetic acid, and added to the medium in a concentration of 10ng/mL (26;27); IGF-1 (GenScript, US) was dissolved in sterile water and used in a concentration of 10nM (28;29) and minoxidil sulfate (Sigma, Germany) was dissolved in dimethyl sulfoxide (DMSO) and added to the medium in a concentration of 5µM (30). In addition, LNA-29a was experimentally used at 2.5µM and 10µM. Controls were: solvent in an exposure time and concentration matched manner; LNA-control for LNA-29a; and unstimulated cells.

Unless otherwise indicated, all cells were stimulated 24h after seeding and then cultured for another 48h. All 4 cell types were stimulated for 2h and 4h with 2.5 or 10µM LNA-29a or LNA-control enriched medium, which was subsequently replaced for non-enriched

medium for a period of 46h and 44h respectively, resulting in total of 21 conditions per cell type. These conditions are summarized in Supplementary Table 1.

Locked nucleic acid design

For miR-29a inhibition, a fluorescent labelled LNA-antimir was used with a phosphorothioated backbone (Exiqon, Denmark), which facilitates cellular uptake without the need for a transfection reagent. The following sequences were used: LNA-29a: 5'-TYE665-ATTTCAGATGGTGCT-3', and LNA-scrambled control: 5'-TYE665-ACGTCTATACGCCCA-3', with TYE665 being the fluorescent label at far-red spectrum (λ=665 nm). The porcine miR-29a sequence was obtained from the miRBase Sequence Database (Release 20). The LNA-control was constructed from a randomized nucleotide sequence that does not bind to microRNAs.

Uptake efficiency of microRNA-29a inhibitor

The effect of concentration and incubation time on the uptake efficiency of the LNA was assessed. Generally, high concentrations of microRNA-inhibitors and long incubation periods are used to maximize its effect. However, for clinical applications, it would be convenient to limit the amount of LNA as this would reduce costs and potentially side effects, while a short incubation time would be desirable for clinical use. On this basis, the uptake efficiencies of the LNA-29a and the LNA-control were evaluated using arterial and venous VSMCs and fibroblasts after 2, 4 and 48h of exposure to 2.5μM and 10μM of fluorescently labelled LNA-29a and LNA-control. Cells were plated in 96-wells plates at a density of 15000 cells/well and incubated for 24h to allow for fully attachment. Cells were cultured for 48h to evaluate LNA uptake efficiency, and received medium enriched with fluorescent labelled LNA in the final 2 or 4h or during the full 48h. In addition, control samples with cells without incubation of LNA were used. To evaluate uptake, samples were fixed in 1% paraformaldehyde and fluorescent signal was measured with flow cytometry (FACS LSRII, BDScience, The Netherlands) where 6000 cells were measured per sample. Data was analyzed using FACSDiva software (BD Bioscience, The Netherlands). Background signal was obtained using control samples of cells that were not incubated with LNA. The mean fluorescence intensity for each concentration and time point was evaluated.

mRNA expression

For all groups, mRNA expression levels of tropoelastin (ELN), fibrillin (FBN1) and lysyl oxidase (LOX) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined. RNA was isolated using Trizol reagent (Invitrogen, The Netherlands). cDNA was synthesized from 1μg total RNA and RT-PCR was performed in duplicate using SYBR Green PCR Mastermix (Biorad, The Netherlands). The expression of each gene was normalized to GAPDH and quantified using delta delta Ct (31). Primer sequences are summarized in Table 1.

Table 1. Primers for qPCR analysis

Gene	Forward primer	Reverse primer
GAPDH	CCACTTTTGATGCTGGGGCT	GGAGATGCTCGGTGTGTTGG
ELN	GGGGCTCCTCTGAAGATGGT	GGCATGGGGAATAGCGACTG
LOX	AGGCGATTTGCCTGTACTGC	GGTGAAATTGTGCAGCCCGA
FBN1	GGCACATGCAGTAACACCGA	TCGGGTTCAGTAGGGCACAG
COL1A1	TAAGGGTGAAGCTGGTCCCC	CACCAGCAATACCAGGAGCG

Protein expression

Cells were lysed with RIPA buffer (50mM Tris-HCl, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1mM EDTA, 1% Triton X-100) containing phosphatase- and protease-inhibitors. A porcine tropoelastin ELISA (Abcam, The Netherlands) was performed and tropoelastin was normalized for total protein per sample.

Statistical analysis

Data is presented as mean +/- standard error of the mean (SEM). For statistical analysis SPSS version 23.0 was used. All conditions were compared with their exposure time and concentration matched control using an unpaired t-test. For FACS data, where 2 groups were compared an unpaired t-test was used, while a one-way ANOVA with Tukey post-hoc test was used for comparing several groups. Various elastin-inducing compound groups for all 4 cell types were compared using a one-way ANOVA with Tukey post-hoc analysis. P-values of <0.05 were considered statistically significant.

RESULTS

Cell isolation

Cells were successfully harvested. Isolated venous and arterial fibroblasts were oblong and triangular shaped. Arterial and venous VSMCs showed a typical elongated shape. Fibroblasts were smaller than VSMCs. Staining for α-SMA and vimentin confirmed that venous and arterial fibroblasts were vimentin+ and α-SMA- while arterial and venous VSMCs were vimentin+ and α-SMA+ (Fig.1).

Tropoelastin mRNA is enhanced by TGF-β but not by IGF-1 and minoxidil sulphate

mRNA transcript levels for ELN, LOX and FBN under all conditions versus their control condition are depicted in Fig.2-4. Incubation with minoxidil sulfate did not result in a significant upregulation of tropoelastin mRNA (Fig.2), whereas IGF-1 only minimally altered this mRNA expression (Fig.2). This therefore did not result in marked increases in tropoelastin protein expression. In contrast, TGF-β substantially increased tropoelastin mRNA levels in all cell types except arterial VSMCs, with an 8.5-fold increase for arterial fibroblasts, 7.8-fold increase in venous fibroblasts and 3-fold increase in venous VSMCs

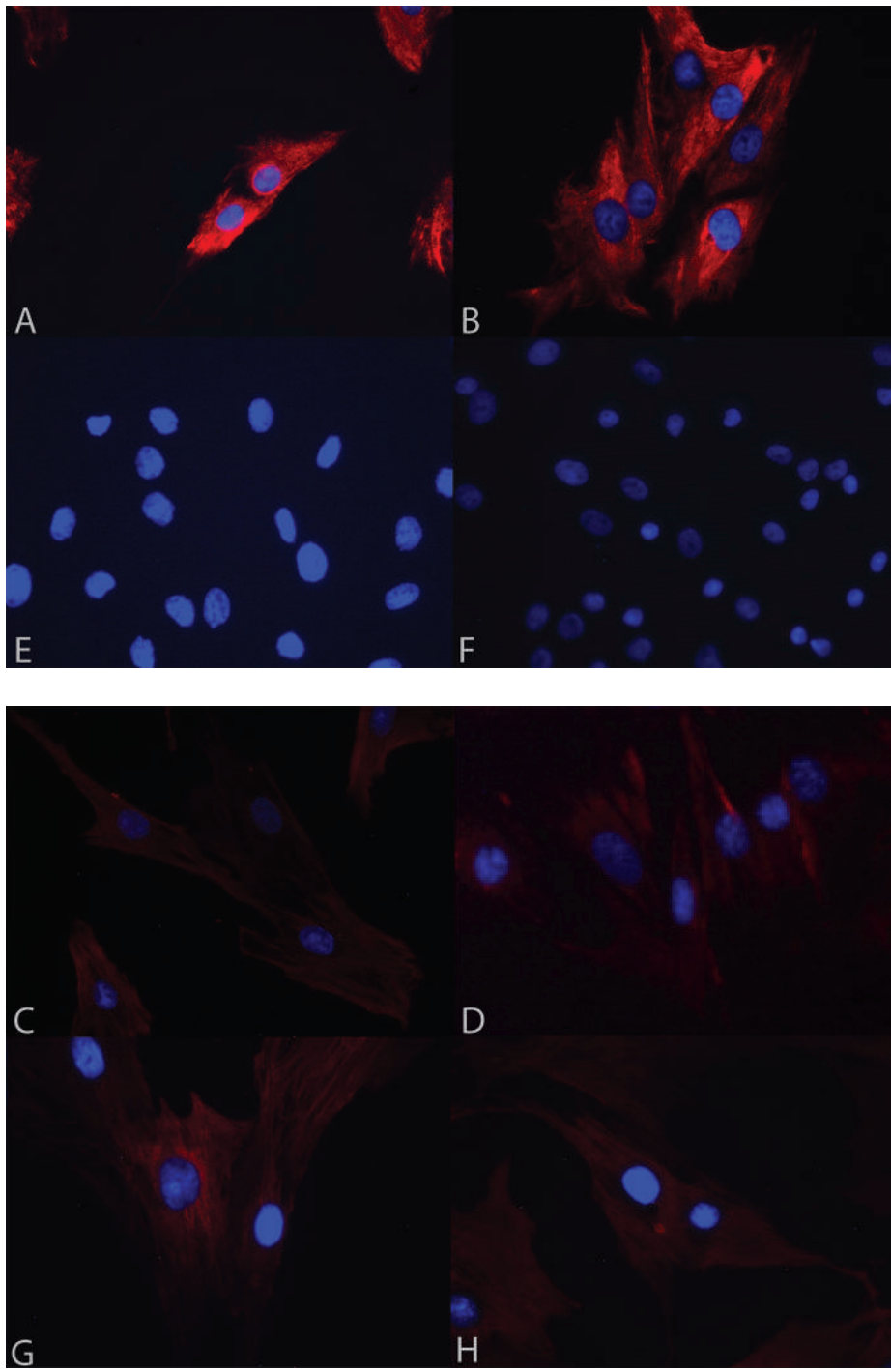


Fig. 1 Vimentin (upper lane) and α-SMA (lower lane) staining of typical cells used in this study. VSMCs were α-SMA and vimentin positive, whereas fibroblasts were vimentin positive but α-SMA negative. A,E: arterial fibroblasts; B,F: venous fibroblasts; C,G: arterial VSMCs; D,H: venous VSMC.

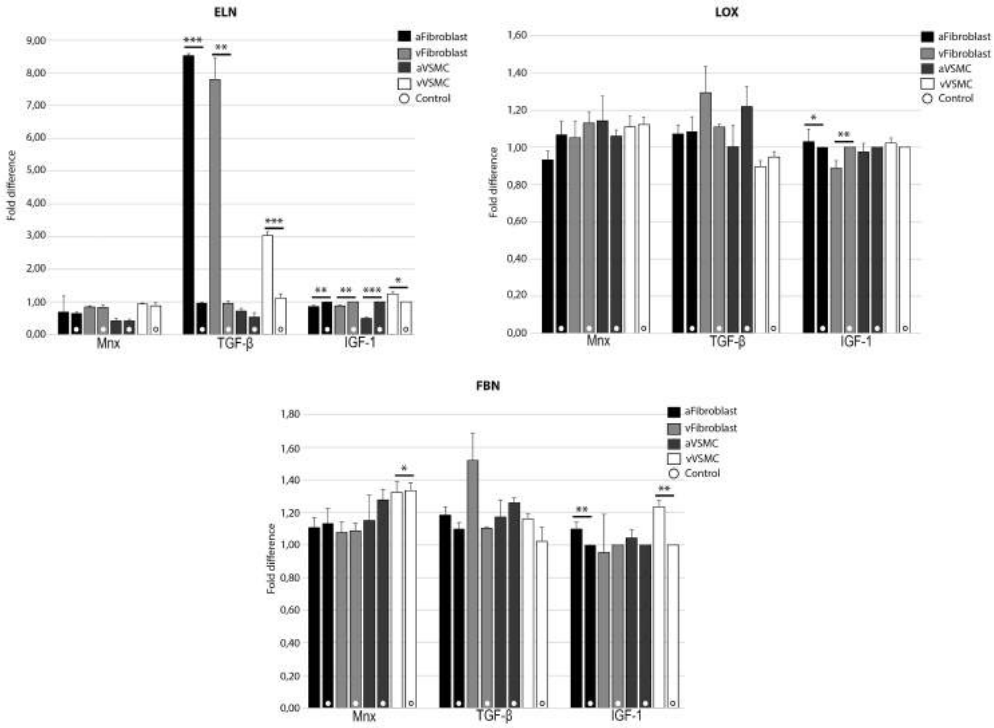


Fig. 2 Mean fluorescent signal representing uptake efficiency of fluorescent-labelled LNA by arterial and venous fibroblasts and VSMCs incubated with 2.5 μM and 10μM LNA in time, as analyzed with FACS. There is a gradual increase in uptake in time, with maximal uptake at 48h. Exposure to high concentrations of LNA (10μM) prompted high uptake. Fluorescent signal was significantly higher for all time points in the 10μM LNA group as compared to the 2.5μM group (2h and 4h p=0.000; 48h p=0.002). In all time points and both concentrations, venous VSMCs had the highest uptake as compared to other cell types. *p<0.05, **p<0.01, *** p<0.001.

(Fig.2). Remarkably, despite this increase in mRNA, this lacked effect on tropoelastin protein levels (Fig.5).

Uptake efficiency of LNA is time and dose dependent

From 2h incubation time onwards, the majority of cells contained LNA-29a although levels varied according to the conditions (Fig.6). Only in the 2.5μM LNA-29a group did a fraction of arterial VSMCs not display any LNA over the time period (data not shown). For both concentrations and all cell types, a gradual increase in uptake was observed over time, with the highest uptake at 48h (Fig.6). Incubation with 10μM resulted in a higher LNA-29a uptake than did 2.5μM. On this basis, the fluorescence signal was significantly higher at all time points in the 10μM LNA-29a group compared to the 2.5μM group (T2h and T4h p<0,001; T48h p=0.002). LNA-control resulted in similar uptake patterns (data not shown).

After 2 h incubation, venous cells contained more LNA-29a than arterial cells at both concentrations (2.5μM p=0.002; 10μM p=0.048), while there was no difference between

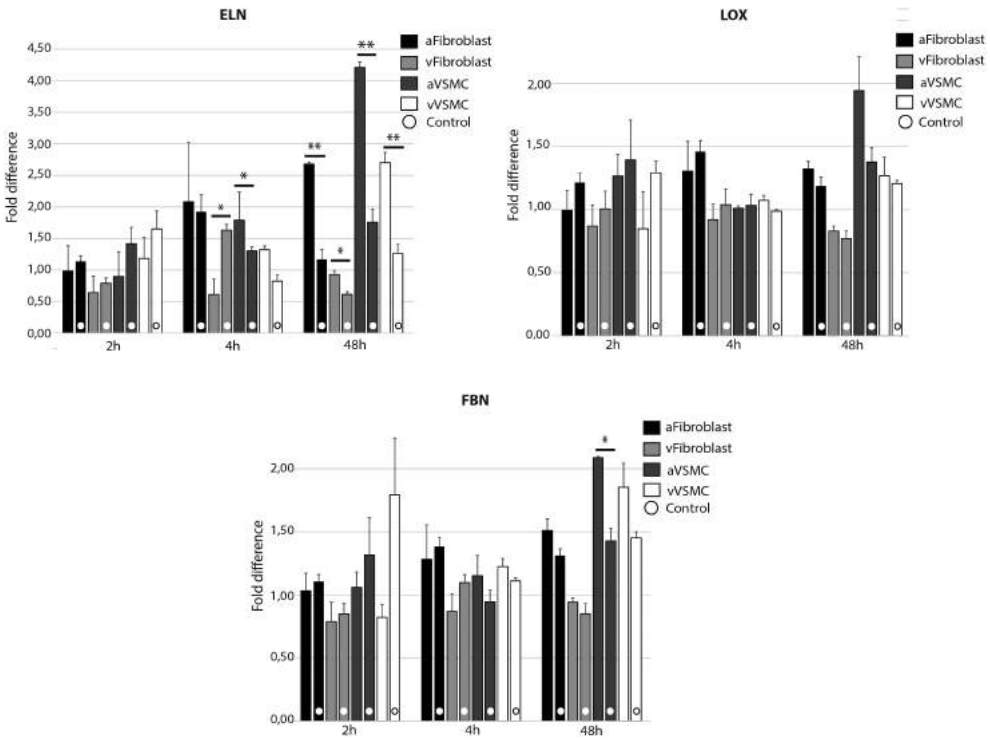


Fig. 3 mRNA expression of tropoelastin (ELN), LOX and FBN for arterial and venous fibroblasts and VSMCs incubated with IGF-1, minoxidil sulfate and TGF-β1 compared to the negative control. *p<0.05, **p<0.01, *** p<0.001.

VSMCs and fibroblasts. This difference disappeared after 4 h of incubation. After 48h, VSMCs showed significantly more LNA-29a uptake than fibroblasts (2.5μM p=0.002; 10μM p<0,001). Except for 2h incubation with 2.5μM LNA-29a, all venous VSMCs displayed a higher uptake than other cell types under similar conditions (Fig.6).

Tropoelastin mRNA is enhanced by LNA-29a

Exposure to 2 concentrations of LNA-29a was evaluated. In parallel with the uptake efficiency patterns (Fig.6), tropoelastin mRNA expression was increasingly enhanced with prolonged incubation (Fig.7). This did not result in significant differences over LNA-control after 2h of incubation with either 2.5μM (Fig.3) or 10μM (Fig.4). Four hours of incubation with 2.5μM LNA significantly promoted tropoelastin mRNA expression in arterial VSMCs (Fig.3), while this was observed in venous VSMCs when incubating with 10μM LNA-29a (Fig.4).

Exposure to LNA-29a for 48h substantially promoted tropoelastin mRNA expression in all cell types when using 2.5μM (Fig.3), and in all cell types except venous fibroblasts when using 10μM. Higher concentrations of LNA-29a resulted in superior tropoelastin mRNA expression levels (Fig.7). The overall highest tropoelastin mRNA levels were induced with

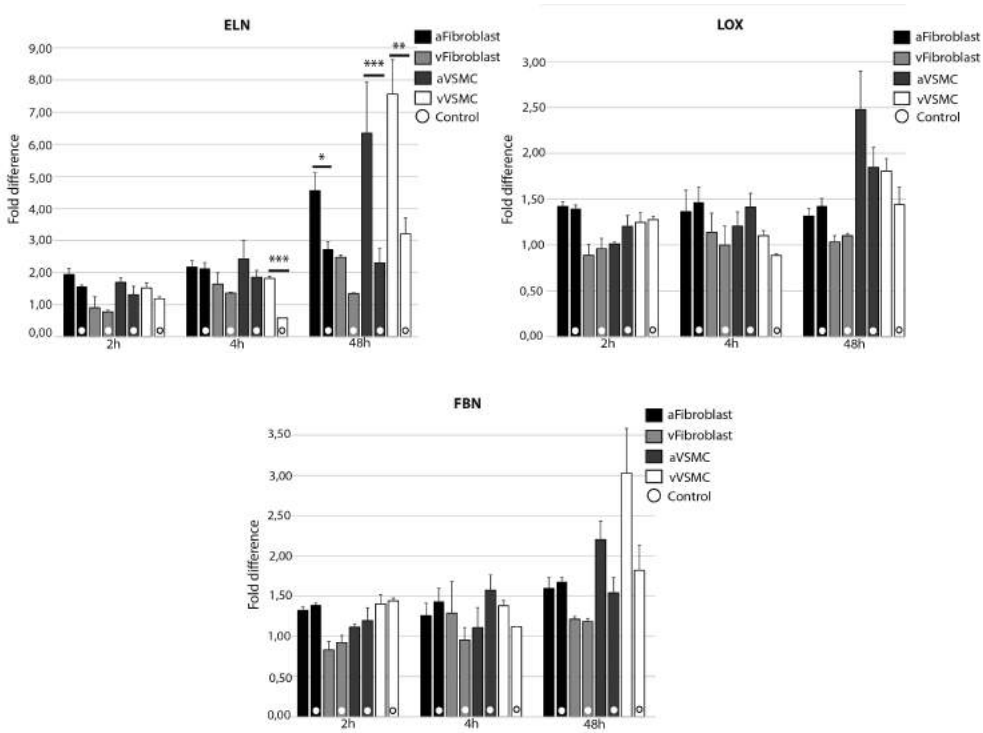


Fig. 4 mRNA expression of ELN, LOX and FBN for arterial and venous fibroblasts and VSMCs incubated with 2.5μM LNA for 2h, 4h and 48h compared to the scrambled control. *p<0.05, **p<0.01.

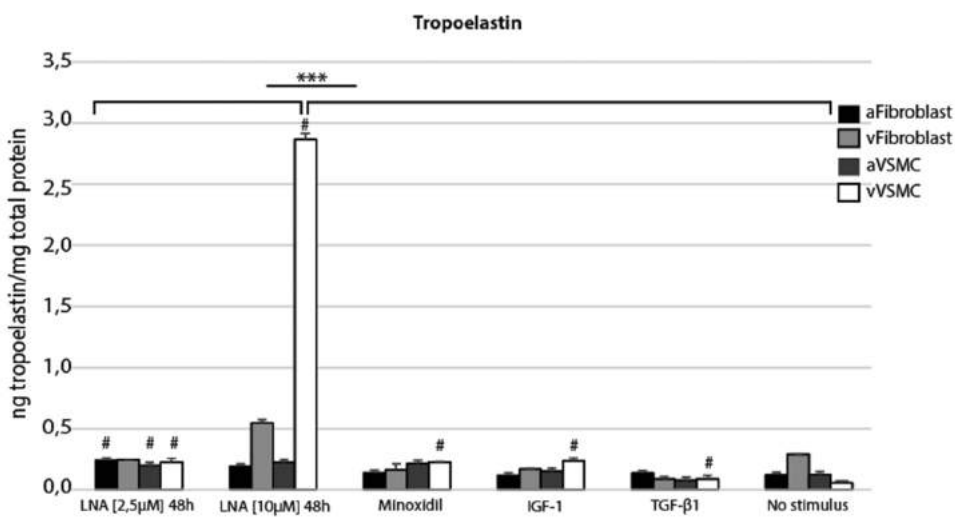


Fig. 5 mRNA expression of ELN, LOX and FBN for arterial and venous fibroblasts and VSMCs incubated with 10μM LNA for 2h, 4h and 48h compared to the scrambled control. *p<0.05, **p<0.01, *** p<0.001.

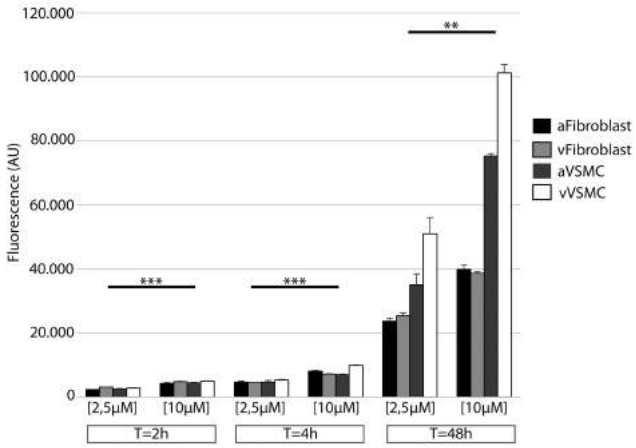


Fig. 6 mRNA expression of ELN for arterial and venous fibroblasts and VSMCs incubated with IGF-1, minoxidil sulfate and TGF-β1 and 2.5 µM and 10µM LNA for 48h. *p<0.05, **p<0.01, *** p<0.001

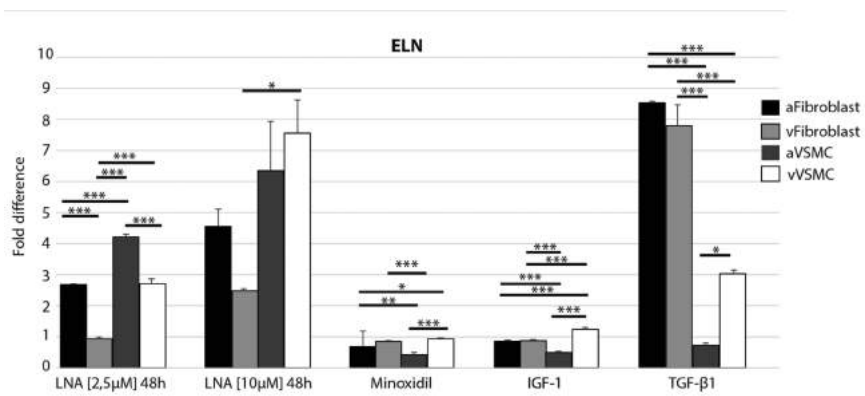


Fig. 7 Tropoelastin levels in ng per mg total protein for arterial and venous fibroblasts and VSMCs incubated with IGF-1, minoxidil and TGF-β1 and 2.5 µM and 10µM LNA for 48h. *** p≤0.001. # significantly different compared to non-stimulated control.

TGF-β in arterial and venous fibroblast, followed by large increases of tropoelastin mRNA in venous and arterial VSMCs incubated with 10µM LNA-29a for 48h (Fig.7).

Tropoelastin protein synthesis is enhanced by LNA-29a in venous VSMCs

Tropoelastin protein levels were significantly higher than their unstimulated controls for venous VSMCs in all conditions and arterial fibroblasts and VSMCs stimulated with 2.5µM LNA-29a and a trend towards arterial VSMCs 10µM LNA29a (Fig.5). However, these differences in protein levels were generally small, except for venous VSMCs 10µM LNA-29a. Overall, when all compounds were compared, these venous VSMCs incubated with 10µM LNA-29a for 48h showed a striking increase in the levels of tropoelastin, which

were significantly higher than the other compounds and cells under comparable conditions ($p<0.001$, Fig.5). This dramatic increase in tropoelastin was in line with the high LNA-29a uptake of venous VSMCs. In addition, venous cells synthesized more tropoelastin than arterial cells ($p=0.011$) and venous VSMCs synthesized more tropoelastin compared to arterial VSMCs ($p=0.011$), arterial fibroblasts ($p=0.010$) and venous fibroblasts ($p=0.049$).

Lysyl oxidase and fibrillin-1 mRNA

In addition to tropoelastin mRNA, lysyl oxidase and fibrillin-1, both involved in elastic fiber formation, were taken into account (Fig.2-4). Only IGF-1 was able to induce very subtle but significant changes in lysyl oxidase mRNA expression in arterial fibroblasts (Fig.2). In addition, incubation with LNA-29a for 48h induced small non-significant upregulation of lysyl oxidase mRNA in the 2.5μM group in all cell types (Fig.3) and larger but non-significant increases in VSMCs in the 10μM group (Fig.4). When all compounds were compared, highest lysyl oxidase mRNA levels were obtained using 10μM LNA-29a incubated for 48h in arterial and venous VSMCs (Supplementary Fig.1).

Fibrillin-1 mRNA was upregulated in arterial fibroblasts and venous VSMCs stimulated with IGF-1 (Fig.2). In addition, incubation with LNA-29a for 48h promoted fibrillin mRNA expression in a dose dependent manner, especially in arterial and venous VSMCs. Only in arterial VSMCs exposed to 2.5μM LNA-29a for 48h, was fibrillin-1 mRNA significantly increased over the scrambled control (Fig.3-4). When all compounds were compared, the largest upregulation of fibrillin-1 mRNA was observed in arterial and venous VSMCs exposed to 10μM for 48h (Supplementary Fig.2).

DISCUSSION

In this study, several elastin-stimulating agents were compared in arterial and venous fibroblasts and VSMCs. These elastogenic agents comprised both positive inducers and an inhibitor of negative regulator miR-29a. Here, we showed that venous VSMCs dramatically increase tropoelastin protein synthesis when exposed to LNA-29a. This appears to be a dose- and incubation time-dependent effect. In contrast, positive inducers IGF-1 and minoxidil sulfate exerted little effect on either tropoelastin mRNA or protein levels, while TGF-β1 induced substantial increases in mRNA expression on arterial fibroblasts and venous fibroblasts and VSMCs but lacked efficacy on protein levels, further underlining the role of post-transcriptional regulation.

miR-29a inhibition

Despite many efforts, elastin remains one of the missing links in vascular tissue engineering(1). It is crucial for compliance of the vascular wall, functions as physical barrier to prevent migration, and plays a role in cellular signaling(5). In contrast to other matrix proteins, tropoelastin is encoded by a single gene, thus supplying a logical target for potential therapeutic strategies to improve elastogenesis. miR-29a has been recently discovered to post-transcriptionally downregulate tropoelastin synthesis by

binding tropoelastin mRNA(24). Although subsequent studies describe its potency on elastogenesis(23;24), until now the effect of miR-29a inhibition has not been compared with positive tropoelastin moderators. In this study, we found that decreasing miR-29a is more potent than enhancing positive inducers of tropoelastin. Furthermore, we identified a miR-29a inhibitor that did not require a transfection reagent and thus is an attractive candidate for use in a clinical setting. The advantageous results of LNA-29a over positive mediators may be explained when considering physiological regulation of elastogenesis during life. Levels of tropoelastin pre-mRNA serve as an indicator of ongoing transcription in rat lungs over time(32); on this basis, pre-mRNA levels increase from low levels in the mid-fetal period to high levels in the neonatal period, paralleled by an increase in the levels of tropoelastin protein. However, in adult tissue, pre-mRNA levels are as high as in the neonatal period, despite 20-fold lower levels of steady-state mRNA. Subsequently, miR-29a was identified as important post-transcriptional regulator of tropoelastin(23). Indeed, miR-29a levels gradually increase with age(23). The high pre-mRNA with low steady-state mRNA levels and increasing expression of miR-29a in adult tissue suggests that miR-29a has a dominant role in the net balance of elastogenesis in adulthood. Thus, this may explain the dramatic increase of tropoelastin protein when miR-29a was blocked as compared with positive tropoelastin mediators.

Differences of miR-29a inhibition per cell type

The effect of miR-29a inhibition on tropoelastin was only observed in venous VSMCs exposed to the high concentration of 10μM LNA-29a for 48h. This may in part be explained by the uptake patterns of LNA-29a. Generally, exposure to high concentration of 10μM LNA-29a resulted in more uptake, tropoelastin mRNA and protein expression than 2.5μM LNA-29a and an exposure time of 48h resulted in more LNA-29a uptake and tropoelastin mRNA expression than 2 or 4 h exposure. In addition, by 48h at either concentration of LNA-29a, VSMCs exhibited more LNA-29a uptake than fibroblasts and venous VSMCs exhibited more LNA-29a uptake than arterial VSMCs. Cell origin and cell type determine cell proliferation, migration, its synthetic profile and its response to chemical and physical stimuli(17;18;20-22;33). Some of these characteristics can be maintained *in vitro*, suggesting a positional memory(22;34). In line with our results, several studies showed that VSMCs are more responsive to tropoelastin enhancing agents than fibroblasts(27;28) and venous VSMCs are often more responsive to drugs than arterial VSMCs(19;35). Venous VSMCs display a more synthetic profile and synthesize more matrix proteins compared to arterial VSMCs(17).

TGF – β1

In addition to miR-29 inhibition, tropoelastin may be enhanced by increasing tropoelastin mRNA expression, by stabilizing tropoelastin mRNA and by decreasing matrix metalloproteinase (MMP)-mediated degradation(5). TGF-β is known to both enhance tropoelastin mRNA expression(36) and stabilization(37) and influence MMP expression(38). In this study, incubation with TGF-β drastically increased tropoelastin mRNA expression.

Despite this increase in mRNA, tropoelastin protein levels were not altered, possibly by post-transcriptional downregulation by miR-29a.

Remarkably and in contrast to the above, TGF-β has also been described to decrease miR-29 expression in fibroblasts(39;40). Although several studies showed that such a TGF-β mediated decrease in miR-29 can decrease collagen mRNA and protein expression(40;41), to the best of our knowledge only one study indirectly evaluated the effect of TGF-β mediated miR-29 decrease on tropoelastin mRNA and failed to show any effects(40). Thus, the amplitude of the TGF-β mediated miR-29 reduction seems to be insufficient to alter tropoelastin levels. Indeed, as mentioned above, in the current experiment we also observed a dose-dependent effect of our miR-29-inhibitor.

Minoxidil sulphate and IGF-1

Minoxidil sulphate, the biologically active variant of minoxidil, has been shown to enhance both tropoelastin mRNA expression and its stability(30), whereas IGF increases tropoelastin mRNA(29). In this study we used concentrations that were reported to be optimal for elastogenesis(28;29)(30), but this did not result in significantly increases in tropoelastin mRNA or protein. Previous studies that did show beneficial effect used embryonic or neonatal cells(28;29;47-49)(42;43), while our studies focused on adult cells. This difference in cell source is very relevant because embryonic cells have a higher elastogenic potential than adult cells(32;44). Because many vascular tissue engineering approaches use adult cells, these studies on elastogenesis with non-adult cells may be less representative.

Cell characterization

In this study, we isolated adventitial fibroblasts and medial VSMCs. The fibroblasts were defined as vimentin+ and α-SMA- cells, whereas α-SMA+ cells were identified as VSMCs(52). However, the latter does not discriminate between contractile VSMCs and synthetic VSMCs or myofibroblasts. However, healthy blood vessels, which were used in this study for cell isolation, are not populated by these myofibroblasts. Thus, the cells isolated from the medial layer were likely mostly contractile VSMCs, but these cells generally dedifferentiate to a more synthetic phenotype in cell culture(53). However, other vascular tissue engineering methods also use isolated medial VSMCs *in vitro*(9) or use cells that are only characterized by α-SMA staining(54-56).

Study limitations

Our study has some potential limitations. First, because of the low levels of intracellular tropoelastin for most conditions after 48h, we did not evaluate extracellular and cross-linked elastin. However, the formation of cross-linked elastic fibers is crucial for elastin functionality. The current study compared various elastogenic stimuli, and form the basis for long-term studies that should also take elastic fiber formation into account. We did evaluate the effect of the tested substances on lysyl oxidase and fibrillin mRNA expression. Lysyl oxidase is involved in crosslinking of tropoelastin whereas fibrillin is a core protein of the microfibril(5). Importantly, LNA-29a also increased lysyl oxidase and fibrillin-1 mRNA.

Secondly, these *in vitro* results should be validated *in vivo*. The activity of proteases such as cathepsins and MMPs, that are in turn are counterbalanced by tissue inhibitors of metalloproteinases (TIMPs), determine the amount of elastin degradation. In addition, although local delivery of LNA-29a may avoid systemic side effects, this should be addressed in *in vivo* studies. Finally, we did not evaluate mechanical stimuli. Flow and cyclic stretch are potent inducers of elastin synthesis(57;58). When pulsatile flow is combined with LNA-29a, this may even further enhance elastogenesis.

CONCLUSION

In conclusion, post-transcriptional downregulation of tropoelastin by miR-29a dominates over positive tropoelastin regulation in adult cells. Venous VSMCs are especially sensitive for antagonizing miR-29a and can dramatically increase tropoelastin mRNA and protein when exposed to LNA-29a.

ACKNOWLEDGEMENTS

T.C.R. and J.R. are supported by the research program of the BioMedical Materials institute (P3.03 DialysisXS), co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation. This study was supported by a grant by the Dutch Kidney Foundation.

REFERENCE LIST

1. Patel A, Fine B, Sandig M, Mequanint K. Elastin biosynthesis: The missing link in tissue-engineered blood vessels. Cardiovasc Res 2006;71:40-9.
2. Rodgers UR, Weiss AS. Cellular interactions with elastin. Pathol Biol (Paris) 2005;53:390-8.
3. Parks WC, Secrist H, Wu LC, Mecham RP. Developmental regulation of tropoelastin isoforms. J Biol Chem 1988;263:4416-23.
4. Davidson JM, Hill KE, Alford JL. Developmental changes in collagen and elastin biosynthesis in the porcine aorta. Dev Biol 1986;118:103-11.
5. Mithieux SM, Weiss AS. Elastin. Adv Protein Chem 2005;70:437-61.
6. Wagenseil JE, Ciliberto CH, Knutsen RH, Levy MA, Kovacs A, Mecham RP. The importance of elastin to aortic development in mice. Am J Physiol Heart Circ Physiol 2010;299:H257-H264.
7. Karnik SK, Brooke BS, Bayes-Genis A, Sorensen L, Wythe JD, Schwartz RS, et al. A critical role for elastin signaling in vascular morphogenesis and disease. Development 2003;130:411-23.
8. Li DY, Brooke B, Davis EC, Mecham RP, Sorensen LK, Boak BB, et al. Elastin is an essential determinant of arterial morphogenesis. Nature 1998;393:276-80.
9. Dahl SL, Kypson AP, Lawson JH, Blum JL, Strader JT, Li Y, et al. Readily available tissue-engineered vascular grafts. Sci Transl Med 2011;3:68ra9.
10. Tschoeke B, Flanagan TC, Koch S, Harwoko MS, Deichmann T, Ella V, et al. Tissue-engineered small-caliber vascular graft based on a novel biodegradable composite fibrin-polylactide scaffold. Tissue Eng Part A 2009;15:1909-18.
11. L'Heureux N, Paquet S, Labbe R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel. FASEB J 1998;12:47-56.
12. Bourget JM, Gauvin R, Larouche D, Lavoie A, Labbe R, Auger FA, et al. Human fibroblast-derived ECM as a scaffold for vascular tissue engineering. Biomaterials 2012;33:9205-13.
13. Wu HC, Wang TW, Kang PL, Tsuang YH, Sun JS, Lin FH. Coculture of endothelial and smooth muscle cells on a collagen membrane in the development of a small-diameter vascular graft. Biomaterials 2007;28:1385-92.
14. Krettek A, Sukhova GK, Libby P. Elastogenesis in human arterial disease: a role for macrophages in disordered elastin synthesis. Arterioscler Thromb Vasc Biol 2003;23:582-7.
15. de Jonge N, Muylaert DE, Fioretta ES, Baaijens FP, Fledderus JO, Verhaar MC, et al. Matrix production and organization by endothelial colony forming cells in mechanically strained engineered tissue constructs. PLoS One 2013;8:e73161.
16. Wise SG, Weiss AS. Tropoelastin. Int J Biochem Cell Biol 2009;41:494-7.
17. Wong AP, Nili N, Strauss BH. In vitro differences between venous and arterial-derived smooth muscle cells: potential modulatory role of decorin. Cardiovasc Res 2005;65:702-10.
18. Gauvin R, Guillemette M, Galbraith T, Bourget JM, Larouche D, Marcoux H, et al. Mechanical properties of tissue-engineered vascular constructs produced using arterial or venous cells. Tissue Eng Part A 2011;17:2049-59.
19. Deng DX, Spin JM, Tsalenko A, Vailaya A, Ben-Dor A, Yakhini Z, et al. Molecular signatures determining coronary artery and saphenous vein smooth muscle cell phenotypes: distinct responses to stimuli. Arterioscler Thromb Vasc Biol 2006;26:1058-65.
20. Lee RM, Masaki T, Yang HS, Liu J, Chen J, Li L, et al. Different signaling responses to anti-proliferative agents in human aortic and venous smooth muscle cells. J Cell Biochem 2006;99:835-44.

21. Parsonage G, Falciani F, Burman A, Filer A, Ross E, Bofill M, et al. Global gene expression profiles in fibroblasts from synovial, skin and lymphoid tissue reveals distinct cytokine and chemokine expression patterns. Thromb Haemost 2003;90:688-97.

22. Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, et al. Diversity, topographic differentiation, and positional memory in human fibroblasts. Proc Natl Acad Sci U S A 2002;99:12877-82.

23. Boon RA, Seeger T, Heydt S, Fischer A, Hergenreider E, Horrevoets AJ, et al. MicroRNA-29 in aortic dilation: implications for aneurysm formation. Circ Res 2011;109:1115-9.

24. Zhang P, Huang A, Ferruzzi J, Mecham RP, Starcher BC, Tellides G, et al. Inhibition of microRNA-29 enhances elastin levels in cells haploinsufficient for elastin and in bioengineered vessels--brief report. Arterioscler Thromb Vasc Biol 2012;32:756-9.

25. Sproul EP, Argraves WS. A cytokine axis regulates elastin formation and degradation. Matrix Biol 2013;32:86-94.

26. Liu JM, Davidson JM. The elastogenic effect of recombinant transforming growth factor-beta on porcine aortic smooth muscle cells. Biochem Biophys Res Commun 1988;154:895-901.

27. Sauvage M, Hinglais N, Mandet C, Badier C, Deslandes F, Michel JB, et al. Localization of elastin mRNA and TGF-beta1 in rat aorta and caudal artery as a function of age. Cell Tissue Res 1998;291:305-14.

28. Rich CB, Ewton DZ, Martin BM, Florini JR, Bashir M, Rosenbloom J, et al. IGF-I regulation of elastogenesis: comparison of aortic and lung cells. Am J Physiol 1992;263:L276-L282.

29. Wolfe BL, Rich CB, Goud HD, Terpstra AJ, Bashir M, Rosenbloom J, et al. Insulin-like growth factor-I regulates transcription of the elastin gene. J Biol Chem 1993;268:12418-26.

30. Slove S, Lannoy M, Behmoaras J, Pezet M, Sloboda N, Lacolley P, et al. Potassium channel openers increase aortic elastic fiber formation and reverse the genetically determined elastin deficit in the BN rat. Hypertension 2013;62:794-801.

31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.

32. Swee MH, Parks WC, Pierce RA. Developmental regulation of elastin production. Expression of tropoelastin pre-mRNA persists after down-regulation of steady-state mRNA levels. J Biol Chem 1995;270:14899-906.

33. Kim SJ, Masaki T, Rowley R, Leypoldt JK, Mohammad SF, Cheung AK. Different responses by cultured aortic and venous smooth muscle cells to gamma radiation. Kidney Int 2005;68:371-7.

34. Van Linthout S, Miteva K, Tschope C. Crosstalk between fibroblasts and inflammatory cells. Cardiovasc Res 2014;102:258-69.

35. Kim SJ, Masaki T, Leypoldt JK, Kamerath CD, Mohammad SF, Cheung AK. Arterial and venous smooth-muscle cells differ in their responses to antiproliferative drugs. J Lab Clin Med 2004;144:156-62.

36. Kahari VM, Olsen DR, Rhudy RW, Carrillo P, Chen YQ, Uitto J. Transforming growth factor-beta up-regulates elastin gene expression in human skin fibroblasts. Evidence for post-transcriptional modulation. Lab Invest 1992;66:580-8.

37. Kuang PP, Zhang XH, Rich CB, Foster JA, Subramanian M, Goldstein RH. Activation of elastin transcription by transforming growth factor-beta in human lung fibroblasts. Am J Physiol Lung Cell Mol Physiol 2007;292:L944-L952.

38. Alvira CM, Guignabert C, Kim YM, Chen C, Wang L, Duong TT, et al. Inhibition of transforming growth factor beta worsens elastin degradation in a murine model of Kawasaki disease. Am J Pathol 2011;178:1210-20.

39. Yang T, Liang Y, Lin Q, Liu J, Luo F, Li X, et al. miR-29 mediates TGFbeta1-induced extracellular matrix synthesis through activation of PI3K-AKT pathway in human lung fibroblasts. J Cell Biochem 2013;114:1336-42.

40. Van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, et al. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A 2008;105:13027-32.

41. Wang B, Komers R, Carew R, Winbanks CE, Xu B, Herman-Edelstein M, et al. Suppression of microRNA-29 expression by TGF-beta1 promotes collagen expression and renal fibrosis. J Am Soc Nephrol 2012;23:252-65.

42. Hayashi A, Suzuki T, Wachi H, Tajima S, Nishikawa T, Murad S, et al. Minoxidil stimulates elastin expression in aortic smooth muscle cells. Arch Biochem Biophys 1994;315:137-41.

43. Tajima S, Hayashi A, Suzuki T, Nishikawa T. Stimulation of elastin expression by minoxidil in chick skin fibroblasts. Arch Dermatol Res 1995;287:494-7.

44. McGowan SE. Influences of endogenous and exogenous TGF-beta on elastin in rat lung fibroblasts and aortic smooth muscle cells. Am J Physiol 1992;263:L257-L263.

45. Tsoporis J, Keeley FW, Lee RM, Leenen FH. Arterial vasodilation and vascular connective tissue changes in spontaneously hypertensive rats. J Cardiovasc Pharmacol 1998;31:960-2.

46. Ruzicka M, Keeley FW, Leenen FH. The renin-angiotensin system and volume overload-induced changes in cardiac collagen and elastin. Circulation 1994;90:1989-96.

47. Rich CB, Goud HD, Bashir M, Rosenbloom J, Foster JA. Developmental regulation of aortic elastin gene expression involves disruption of an IGF-I sensitive repressor complex. Biochem Biophys Res Commun 1993;196:1316-22.

48. Li J, Masood A, Yi M, Lau M, Belcastro R, Ivanovska J, et al. The IGF-I/IGF-R1 pathway regulates postnatal lung growth and is a nonspecific regulator of alveologenesis in the neonatal rat. Am J Physiol Lung Cell Mol Physiol 2013;304:L626-L637.

49. Badesch DB, Lee PD, Parks WC, Stenmark KR. Insulin-like growth factor I stimulates elastin synthesis by bovine pulmonary arterial smooth muscle cells. Biochem Biophys Res Commun 1989;160:382-7.

50. Chen Y, Capron L, Magnusson JO, Wallby LA, Arnqvist HJ. Insulin-like growth factor-1 stimulates vascular smooth muscle cell proliferation in rat aorta in vivo. Growth Horm IGF Res 1998;8:299-303.

51. Kothapalli CR, Ramamurthi A. Benefits of concurrent delivery of hyaluronan and IGF-1 cues to regeneration of crosslinked elastin matrices by adult rat vascular cells. J Tissue Eng Regen Med 2008;2:106-16.

52. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 2004;84:767-801.

53. Worth NF, Rolfe BE, Song J, Campbell GR. Vascular smooth muscle cell phenotypic modulation in culture is associated with reorganisation of contractile and cytoskeletal proteins. Cell Motil Cytoskeleton 2001;49:130-45.

54. Brennan MP, Dardik A, Hibino N, Roh JD, Nelson GN, Papademitris X, et al. Tissue-engineered vascular grafts demonstrate evidence of growth and development when implanted in a juvenile animal model. Ann Surg 2008;248:370-7.

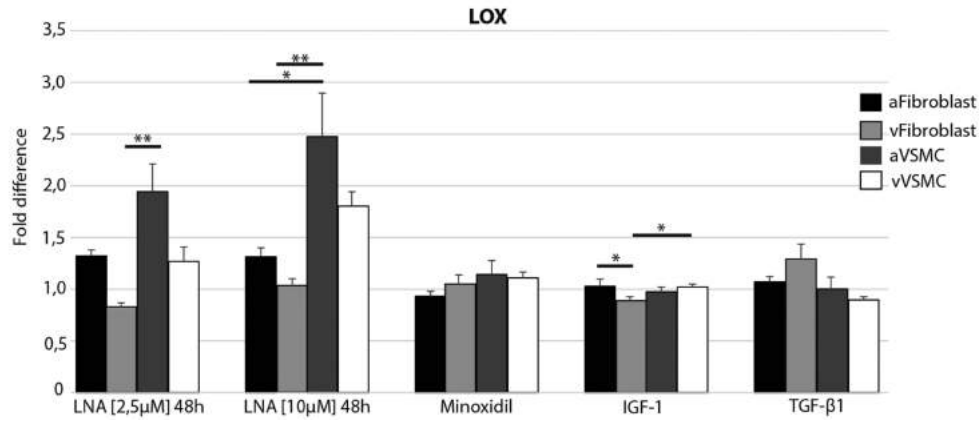
55. de Valence S, Tille JC, Mugnai D, Mrowczynski W, Gurny R, Moller M, et al. Long term performance of polycaprolactone vascular grafts in a rat abdominal aorta replacement model. Biomaterials 2012;33:38-47.

56. Syedain ZH, Meier LA, Lahti MT, Johnson SL, Tranquillo RT. Implantation of completely biological engineered grafts following decellularization into the sheep femoral artery. Tissue Eng Part A 2014;20:1726-34.

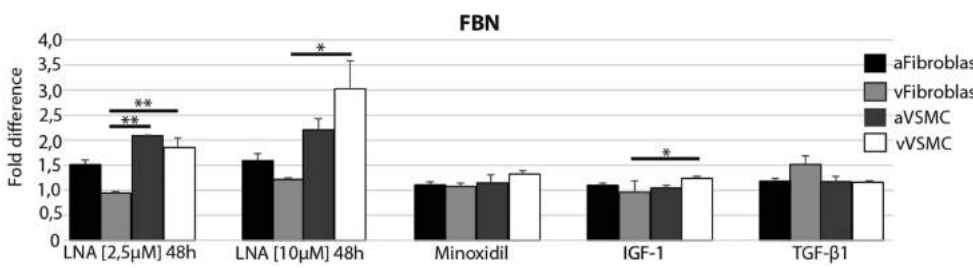
57. Venkataraman L, Bashur CA, Ramamurthi A. Impact of cyclic stretch on induced elastogenesis within collagenous conduits. Tissue Eng Part A 2014;20:1403-15.

58. Ben DA, Benessiano J, Poitevin P, Levy BI, Michel JB. Arterial expansive remodeling induced by high flow rates. Am J Physiol 1997;272:H851-H858.

SUPPLEMENTARY FIGURES AND TABLES



Suppl. fig. 1. mRNA expression of FBN for arterial and venous fibroblasts and VSMCs incubated with IGF-1, minoxidil and TGF-β1 and 2.5 µM and 10µM LNA for 48h.



Suppl. fig. 2. mRNA expression of LOX for arterial and venous fibroblasts and VSMCs incubated with IGF-1, minoxidil and TGF-β1 and 2.5 µM and 10µM LNA for 48h.

Suppl. table 1. Experimental conditions used for arterial and venous VSMCs and fibroblasts. All cells were cultured for a total period of 48h.

Compound	Solvent	Experimental concentration	Exposure time	Control
TGF-β1	Acetid acid	10ng/mL	48h	Acetid acid
IGF-1	Sterile water	10nM	48h	Unstimulated
Minoxidil sulfate	DMSO	5μM	48h	DMSO
LNA-29a	PBS	2.5 μM	48h	LNA-control
LNA-29a	PBS	10 μM	48h	LNA-control
LNA-29a	PBS	2.5 μM	2h	LNA-control
LNA-29a	PBS	10 μM	2h	LNA-control
LNA-29a	PBS	2.5 μM	4h	LNA-control
LNA-29a	PBS	10 μM	4h	LNA-control
Unstimulated	-	-	48h	-