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METHODS ARTICLE

Promoting Tropoelastin Expression in Arterial and Venous Vascular Smooth Muscle Cells and Fibroblasts for Vascular Tissue Engineering

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Elastin, critical for its structural and regulatory functions, is a missing link in vascular tissue engineering. Several elastin-inducing compounds have previously been reported, but their relative efficiency in promoting elastogenesis by adult arterial and venous vascular smooth muscle cells (VSMCs) and fibroblasts, four main vascular and elastogenic cells, has not been described. In addition to elasto-inductive substances, microRNA-29a was recently established as a potent post-transcriptional inhibitor of elastogenesis. Here, we explored if stimulating positive regulators or blocking inhibitors of elastogenesis could maximize elastin production. We tested whether the elasto-inducing compounds IGF-1, TGF- β 1, and minoxidil could indeed augment elastin production, and whether microRNA-29a antagonism could block elastin production in adult arterial and venous fibroblasts and VSMCs. The effects on elastin, lysyl oxidase, and fibrillin-1 mRNA expression levels and tropoelastin protein were determined. IGF-1 and minoxidil exerted little effect on tropoelastin mRNA expression levels in all cell types, while TGF- β 1 predominantly enhanced mRNA tropoelastin levels, but this mRNA increase did not impact tropoelastin protein abundance. In contrast, microRNA29a inhibition resulted in the upregulation of tropoelastin mRNA in all cell types, but most pronounced in venous VSMCs. Importantly, microRNA-29a-antagonism also enhanced lysyl oxidase and fibrillin-1 mRNA expression, and revealed a dose-dependent increase in tropoelastin protein expression in venous VSMCs. Our studies suggest that the elastogenic potential of microRNA-29a inhibition in vascular cells is superior to that of established elastin-stimulating compounds IGF-1, TGF- β 1, and minoxidil. Thus, microRNA-29a antagonism could serve as an attractive means of enhancing elastin synthesis in tissue-engineered blood vessels.

Introduction

TISSUE-ENGINEERED BLOOD VESSELS (TEBVs) hold great promise as vascular substitutes. Despite this potential, the formation of functional elastic fibers remains a major challenge for TEBVs.¹ Tropoelastin is secreted by cells of mesenchymal origin into the extracellular space as a soluble tropoelastin monomer. It is subsequently oxidized by lysyl oxidase and deposited as a cross-linked protein onto fibrillin-rich microfibrils that lead to the formation of mature elastin fibers.² Importantly, elastogenesis primarily occurs

during the late fetal and early neonatal periods, and is notably absent in mature tissues.^{3,4} As such, generating elastin-rich TEBVs to date 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 resilience and compliance of the vessel.^{5,6} Next to the mechanical properties of the elastic lamellae, they also form a critical structural barrier that effectively prevents adventitial fibroblasts and medial vascular smooth muscle cells (VSMCs) from migrating into the intimal region in response to injury. Furthermore, elastin signaling is an effective

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repressor of VSMC proliferation and migration.⁷ The biological consequences of tropoelastin deficiency are clearly illustrated in the tropoelastin knockout mouse, which dies prematurely as a result of severe intimal hyperplasia.⁸ Collectively, these findings suggest that the incorporation of elastic fibers is likely important for the generation of TEBVs with adequate vascular function.

In vascular tissue engineering, both VSMCs and fibroblasts derived from adult arteries and veins are frequently used as the main cell sources.^{9–13} Although tropoelastin can be synthesized by several cell types,^{14,15} the aforementioned cells remain the primary elastogenic cells.¹⁶ While the biological consequences of numerous elastogenic compounds have been tested in a variety of cell types, their efficiency to induce tropoelastin in these four cell types is yet to be compared side-by-side. This issue is important, as there are marked differences in phenotype, function, and response to physical and chemical stimuli between arterial and venous cells, as well as individually between VSMCs and fibroblasts.^{17–22} Recent work has identified that microRNA-29a (miR-29a) functions as a post-transcriptional inhibitor of tropoelastin synthesis.^{23,24} Since both tropoelastin mRNA inductive and miR-29a inhibiting agents influence tropoelastin production in various cell types,^{24,25} we aimed to determine which method is the most efficacious in increasing elastogenesis by vascular cells. Therefore, we assessed whether treatment with IGF-1, TGF- β 1, minoxidil sulfate, or miR-29a antagonism could differentially influence mRNA and protein levels of established elastogenic markers in arterial and venous VSMCs and fibroblasts.

Materials and Methods

Cell isolation

Medial VSMCs and adventitial fibroblasts were isolated from carotid arteries and jugular veins from female Dutch Landrace adult pigs. To obtain the fibroblasts, the adventitia was stripped from the medial layer, minced, and incubated with collagenase A (Sigma-Aldrich, Germany) for 3 h. After centrifuging the suspension at 1500 rpm for 5 min, the cell pellet was resuspended in the fibroblast medium and seeded onto culture dishes. 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 removed using tweezers, and the tissue sections, containing solely the medial layer, were cut into small pieces. These pieces were pinned with 25G needles to the bottom of a Petri dish with the luminal side facing downward 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 (1:1000; Immunologic, The Netherlands) and α -smooth muscle actin (SMA, 1:1000; Dako, The Netherlands) and secondary antibodies labeled 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

Arterial and venous VSMCs were cultured in M199 (PAA/GE Healthcare, Austria) supplemented with heat inactivated 10% fetal calf serum, 1% penicillin/streptomycin (PAA/GE Healthcare, Austria), 0.25 μ g/mL amphotericin B (Gibco, LifeTech, The Netherlands), 10 mM HEPES (PAA/GE Healthcare, Austria), and 4 mM L-glutamine (PAA/GE Healthcare, Austria). Arterial and venous fibroblasts were cultured in DMEM (PAA/GE Healthcare, Austria) supplemented with heat inactivated 10% fetal calf serum, 1% penicillin/streptomycin (PAA/GE Healthcare, Austria), 0.25 μ g/mL amphotericin B (Gibco, LifeTech, The Netherlands), 1 mM sodium pyruvate (PAA/GE Healthcare, Austria), and 2 mM L-glutamine (PAA/GE Healthcare, Austria). All cells were incubated at 37°C and 5% CO₂. For experiments described in this article, cells of passage 4–5 were generally used.

Experimental conditions

For each of the four cell types (arterial and venous VSMCs and fibroblasts), four different elastogenic stimuli were evaluated: TGF- β 1, minoxidil sulfate, IGF-1, and a miR-29a inhibitor (locked nucleotide anti-miR [LNA]-29a). TGF- β 1 (LifeTech, Germany) was dissolved in acetic acid and added to the medium in a concentration of 10 ng/mL^{26,27}; IGF-1 (GenScript) was dissolved in sterile water and used in a concentration of 10 nM,^{28,29} and minoxidil sulfate (Sigma, Germany) was dissolved in dimethyl sulfoxide (DMSO) and added to the medium in a concentration of 5 μ M.³⁰ In addition, LNA-29a was experimentally used at 2.5 and 10 μ M. For the described experiments, controls were also performed and made use of solvent in which the compound was diluted for similar exposure time, while an LNA-control was utilized (as described below) for LNA-29a. Also, for several experiments, unstimulated cells were utilized.

Unless otherwise indicated, all cells were stimulated 24 h after seeding and then cultured for another 48 h. All four cell types were stimulated for 2 and 4 h with 2.5 or 10 μ M LNA-29a or LNA-control-enriched medium, which was subsequently replaced with nonenriched medium for a period of 46 and 44 h, respectively, resulting in total of 21 conditions per cell type. These conditions are summarized in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/tec).

Locked nucleic acid design

For miR-29a inhibition, a fluorescent labeled 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-ATTTTCAGATGGTGTCT-3' and LNA-scrambled control: 5'-TYE665-ACGTCTATACGCCCA-3', with TYE665 being the fluorescent label at the 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 miRs.

Uptake efficiency of microRNA-29a inhibitor

The effect of concentration and incubation time on the uptake efficiency of the LNA was assessed. Generally, high

TABLE 1. PRIMERS FOR qPCR ANALYSIS

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

qPCR, quantitative PCR.

concentrations of miR 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 48 h of exposure to 2.5 and 10 μ M of fluorescently labeled LNA-29a and LNA-control. Cells were plated in 96-well plates at a density of 15,000 cells/well and incubated for 24 h to allow for full attachment. Cells were cultured for 48 h to evaluate LNA uptake efficiency and received a medium enriched with fluorescent labeled LNA in the final 2 or 4 h or during the full 48 h. 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 were 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, fibrillin-1, and lysyl oxidase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined. RNA was iso-

lated using the TRIzol reagent (Invitrogen, The Netherlands). cDNA was synthesized from 1 μ g total RNA and real time 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.

Protein expression

Cells were lysed with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM 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 are presented as mean $\pm$ standard error of the mean. 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 two 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 tropoelastin-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.

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. SMA, smooth muscle actin; VSMC, vascular smooth muscle cell. Color images available online at www.liebertpub.com/tec

FIG. 2. mRNA expression of tropoelastin (ELN), lysyl oxidase (LOX), and fibrillin-1 (FBN) for arterial and venous fibroblasts and VSMCs incubated with IGF-1, minoxidil sulfate (Mnx), and TGF- β 1 compared to the negative control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; aFibroblast, arterial fibroblast; vFibroblast, venous fibroblast; aVSMC, arterial vascular smooth muscle cell; vVSMC, venous vascular smooth muscle cell.

Results

Characterization of arterial and venous VSMCs and fibroblasts

While arterial and venous VSMCs displayed their typically elongated morphology, arterial and venous fibroblasts displayed an oblong and triangular morphology. Interestingly, the isolated fibroblasts were markedly smaller than VSMCs. Immunohistochemical analysis using α -SMA and vimentin enabled us to confirm VSMC (vimentin⁺/ α -SMA⁺) and fibroblast (vimentin⁺/ α -SMA⁻) identity (Fig. 1).

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

Using arterial and venous VSMCs and fibroblasts, we investigated to which degree established tropoelastin inducers could stimulate the tropoelastin production of this critical protein for physiologic vessel function, namely TGF- β , IGF-1, and minoxidil sulfate. In addition to tropoelastin mRNA levels, we also assessed the expression levels of lysyl oxidase and fibrillin-1 under these conditions, proteins that play pivotal roles in elastic fiber formation (Fig. 2).

RNA harvested from cells stimulated for 48 h revealed markedly increased expression of tropoelastin mRNA transcript levels following TGF- β treatment, with in particular an 8.5-fold increase in tropoelastin mRNA levels observed in arterial fibroblasts, and 3.0- and 7.8-fold increases observed in venous VSMCs and fibroblasts, respectively (Fig. 2). Remarkably, despite these striking increases in tropoelastin mRNA following TGF- β treatment, we did not observe this increase in tropoelastin protein expression (Fig. 3). While IGF-1 was found to slightly impact expression of tropoelastin mRNAs (Fig. 2), minoxidil sulfate treatment did not appear to impact tropoelastin, lysyl oxidase, or fibrillin-1 mRNA expression (Fig. 2). Of note, IGF-1 was able to induce very subtle but significant changes in lysyl oxidase mRNA expression in arterial fibroblasts (Fig. 2), as well as fibrillin-1 mRNA levels in arterial fibroblasts and venous VSMCs (Fig. 2). Tropoelastin protein levels were only minimally enhanced in venous VSMCs when stimulated with IGF-1 or minoxidil sulfate (Fig. 3).

Uptake efficiency of LNA is time and dose dependent

Having identified that compounds with well-established tropoelastin-stimulating properties were unable to augment tropoelastin protein production in arterial and venous

VSMCs and fibroblasts, we used another strategy, namely inhibition of miR-29a. For this, we designed an LNA that specifically targeted miR-29a, named LNA-29a. First, we tested the uptake kinetics of the compound, while also controlling for visible signs of toxicity based on concentration effects. Within 2 h, it was clear that the majority of cells contained LNA-29a, although uptake varied considerably according to the conditions (Fig. 4). A gradual increase in uptake was observed over time, which we found was maximal at 48 h (Fig. 4). Incubation with 10 μ M resulted in a higher LNA-29a uptake than did 2.5 μ M. On this basis, the fluorescence signal was significantly higher at all time points in the 10 μ M LNA-29a group compared to the 2.5 μ M group (T2 h and T4 h $p < 0.001$; T48 h $p = 0.002$). LNA-control resulted in similar uptake patterns (data not shown). After 48 h, VSMCs showed significantly more LNA-29a uptake than fibroblasts (2.5 μ M $p = 0.002$; 10 μ M $p < 0.001$).

Tropoelastin mRNA is enhanced by LNA-29a

Exposure to 2.5 and 10 μ M LNA-29a enhanced tropoelastin mRNA expression in a time- and dose-dependent manner (Figs. 5 and 6). While significant changes in tropoelastin mRNA levels were not observed between LNA-29a- and LNA-control-treated VSMCs and fibroblasts within 2 h of treatment (Figs. 5 and 6), after 4 h of treatment, 2.5 μ M

FIG. 3. Tropoelastin levels in nanogram per milligram total protein for arterial and venous fibroblasts and VSMCs incubated with IGF-1, minoxidil, and TGF- β 1 and 2.5 and 10 μ M LNA for 48 h. *** $p \leq 0.001$. #significantly different compared to nonstimulated control. LNA, locked nucleotide anti-miR.

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

LNA-29a significantly promoted tropoelastin mRNA expression in arterial VSMCs (Fig. 5), while this effect was observed in venous VSMCs when incubating with 10 μM LNA-29a (Fig. 6).

Exposure of arterial and venous VSMCs and fibroblasts to LNA-29a for 48 h substantially promoted tropoelastin mRNA expression in all cell types when using 2.5 μM (Fig. 5), and in all cell types except venous fibroblasts when using 10 μM (Fig. 6). Higher concentrations of 10 μM LNA-29a resulted in superior tropoelastin mRNA expression levels (Fig. 7). The tropoelastin mRNA levels were found to be maximally induced by TGF- β (Fig. 7), 10 μM LNA-29a treatment also led to striking increases in tropoelastin mRNA in both venous and arterial VSMCs (Fig. 7).

We also investigated the capacity of LNA-29a treatment to impact lysyl oxidase mRNA expression levels in arterial and venous VSMCs and fibroblasts. As shown in Figure 5, 48-h exposure to 2.5 μM LNA-29a induced small, nonsignificant increases in lysyl oxidase mRNA levels, while treatment with

10 μM LNA-29a resulted in larger, yet nonsignificant increases in lysyl oxidase mRNA levels in VSMCs (Fig. 6). On comparing all treatment conditions, lysyl oxidase mRNA levels were found to be increased most following 48-h treatment with 10 μM LNA-29a incubated for 48 h in arterial and venous VSMCs (Supplementary Fig. S1).

Finally, LNA-29a treatment for 48 h promoted fibrillin-1 mRNA expression in a dose-dependent manner, in particular in arterial and venous VSMCs. However, only arterial VSMCs exposed to 2.5 μM LNA-29a for 48 h displayed a significant increase in mRNA levels of fibrillin-1 compared to the LNA-scrambled control (Figs. 5 and 6). The largest upregulation of fibrillin-1 mRNA was observed in arterial and venous VSMCs exposed to 10 μM LNA-29a for a duration of 48 h (Supplementary Fig. S2).

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

As previously mentioned, despite the large increase in tropoelastin mRNA expression levels following TGF- β treatment, an increase in tropoelastin protein was not observed. Therefore, having observed significant increases in tropoelastin mRNA in LNA-29a-treated arterial and venous VSMCs, we assessed tropoelastin protein levels following these treatments in cell lysates using an ELISA. As shown in Figure 3, tropoelastin protein levels were significantly higher than unstimulated controls for venous VSMCs in all conditions, as well as in arterial VSMCs and fibroblasts stimulated with 2.5 μM LNA-29a. Furthermore, a trend toward increased tropoelastin protein production was observed in arterial VSMCs treated with 10 μM LNA-29a for 48 h (Fig. 3). However, it should be noted that aside from venous VSMCs treated with 10 μM LNA-29a, these differences in tropoelastin protein levels were generally small. Collectively, only venous VSMCs treated with 10 μM LNA-29a for 48 h showed a striking increase in the levels of tropoelastin, a finding that is in keeping with the high LNA-29a uptake in these cells. Comparatively, venous cells synthesized more tropoelastin than arterial cells ($p=0.011$), while venous VSMCs synthesized more tropoelastin than arterial VSMCs ($p=0.011$), arterial fibroblasts ($p=0.010$), and venous fibroblasts ($p=0.049$).

Discussion

In this study, the capacity of several compounds to stimulate tropoelastin production in arterial and venous VSMCs and fibroblasts was compared. In these settings,

FIG. 5. mRNA expression of ELN, LOX, and FBN for arterial and venous fibroblasts and VSMCs incubated with 2.5 μM LNA for 2, 4, and 48 h compared to the scrambled control. * $p<0.05$, ** $p<0.01$.

FIG. 6. mRNA expression of ELN, LOX, and FBN for arterial and venous fibroblasts and VSMCs incubated with 10 μ M LNA for 2, 4, and 48 h compared to the scrambled control. * p < 0.05, ** p < 0.01, *** p < 0.001.

TGF- β 1 was found to substantially induce tropoelastin mRNA expression in VSMCs and fibroblasts, but displayed an inability to enhance tropoelastin protein levels. Moreover, IGF-1 and minoxidil sulfate exerted little effect on either tropoelastin mRNA or protein levels. In contrast, we identified that antagonism of miR-29a, via LNA-29a treatment, induces tropoelastin mRNA and protein production in venous VSMCs in a dose- and time-dependent manner. Collectively, these studies implicate a role for post-transcriptional processing of tropoelastin mRNA as a critical influencer of mRNA stability and translation efficiency.

Despite many efforts to date, the elaboration of elastin in vascular tissue engineering approaches has remained problematic.¹ Besides its structural and mechanical role in the vascular wall, it influences VSMC phenotype and functions as physical barrier to prevent VSMC and fibroblast migration.⁵ In contrast to other matrix proteins, tropoelastin is encoded by a single gene, enhancing the development of therapeutic strategies designed to improve elastogenesis.⁵ Recently, miR-29a was discovered to decrease tropoelastin synthesis post-transcriptionally, by binding to the tropoelastin mRNA.²⁴ Although subsequent studies described a potent effect on elastogenesis,^{23,24} the consequences of miR-29a inhibition in comparison to established inducers of tropoelastin production had not been investigated. Our studies identified that blocking miR-29a influences tropoelastin production more strongly than enhancing inducers of tropoelastin synthesis. Furthermore, our utilization of LNA-29a to effectively block miR-29a does not require a transfection reagent, making this approach attractive 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. Elastogenesis peaks

in the neonatal period.³² However, in adult cells, tropoelastin pre-mRNA levels are comparably high to neonatal cells, despite 20-fold lower levels of steady-state tropoelastin mRNA and thus low protein synthesis in adult cells³² due to miR-29a post-transcriptional downregulation.²³ This suggests that miR-29a has a dominant role in the net balance of elastogenesis in adulthood. Indeed, miR-29a levels gradually increase with age.²³ This could potentially explain the dramatic increase of tropoelastin protein when miR-29a was blocked compared with positive tropoelastin mediators. In addition, this could explain the minimal effects of minoxidil sulfate and IGF-1 when compared to other studies, where IGF-1 and minoxidil sulfate were evaluated in especially embryonic or neonatal cells^{25,28–30,33–36} with a high elastogenic potential³² and low miR-29a levels²³ compared to adult cells.

The effect of miR-29a inhibition on tropoelastin was only observed in venous VSMCs exposed to the high concentration of 10 μ M LNA-29a for 48 h. This may, in part, be explained by the uptake patterns of LNA-29a. Generally, exposure to high concentration of 10 μ M LNA-29a resulted in more uptake and tropoelastin mRNA and protein expression than 2.5 μ M LNA-29a, and an exposure time of 48 h resulted in more LNA-29a uptake and tropoelastin mRNA expression than a 2- or 4-h exposure. In addition, by 48 h 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.^{17,18,20–22,37} Some of these characteristics can be maintained *in vitro*, suggesting a positional memory.^{22,38} In line with our results, several studies showed that VSMCs are

FIG. 7. mRNA ELN for arterial and venous fibroblasts and VSMCs incubated with IGF-1, minoxidil sulfate, and TGF- β 1 and 2.5 and 10 μ M LNA for 48 h. * p < 0.05, ** p < 0.01, *** p < 0.001.

more responsive to tropoelastin enhancing agents than fibroblasts^{27,28} and venous VSMCs are often more responsive to drugs than arterial VSMCs.^{19,39} Venous VSMCs display a more synthetic profile and synthesize more matrix proteins compared to arterial VSMCs.¹⁷

In addition to miR-29a 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 expression⁴⁰ and stabilization⁴¹ and influence MMP expression.⁴² 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 the above-mentioned post-transcriptional regulation.

Study limitations

While our study details the successful elaboration of tropoelastin following LNA-29a treatment in VSMCs and fibroblasts, we did not evaluate extracellular and cross-linked elastin. However, the formation of cross-linked elastic fibers is crucial for elastin functionality. As such, our evaluation of various elastogenic stimuli, and identification that solely LNA-29a treatment augments tropoelastin protein production, could form the basis for long-term *in vivo* studies that could further investigate whether this treatment could also enhance elastic fiber formation. It should be noted that LNA-29a treatment also increased lysyl oxidase and fibrillin-1 mRNA, and given that lysyl oxidase is involved in tropoelastin crosslinking, and fibrillin serves as a core protein in microfibrils,⁵ our data suggest that LNA-29a could also improve elastic fiber formation. The before mentioned long-term *in vivo* studies also enable to study the combined effect of LNA-29a and mechanical stimuli. Since flow and cyclic stretch are potent inducers of tropoelastin synthesis,^{43,44} the implementation of pulsatile flow in combination with LNA-29a treatment may further enhance elastogenesis.

The question remains if a single dose of LNA-29a would be sufficient for these long-term studies. We did not perform experiments longer than 48 h and therefore did not evaluate the half-life time of LNA-29a. However, LNA has a fully phosphorothioate modified backbone, which makes it highly resistant to enzymatic degradation. Other groups have performed experiments using LNA-29 with longer term follow-up but other endpoints. These studies demonstrated an almost complete downregulation of microRNA-29 at 1 week²³ and even 4 weeks⁴⁵ after a single dose of intravenous injection in mice. In contrast to those studies where LNA was administered systemically, for vascular tissue engineering purposes, the most obvious method of LNA-29 application would be locally, to avoid potential systemic side effects. Although local side effects of LNA-29a in the vascular wall seem less likely because endothelial cells and monocytes express only little amounts of microRNA-29 in comparison to VSMCs,²³ future *in vivo* studies should evaluate potential side effects on the vascular wall.

Last, the current experiments are performed with porcine cells. Porcine models are frequently used to evaluate tissue-engineered grafts^{46–49} due to their comparable anatomy and vascular response to humans.⁵⁰ Importantly, microRNA-29a

is completely conserved between humans and pigs. Although ssc-miR-29a (sus scrofa, porcine) has one extra nucleotide compared to has-miR-29a (homo sapiens, human), the seed sequence and thus the targets are identical (miR-base). On this basis, it is not expected that there is a species difference, which enables future extrapolation to humans.

Conclusion

Of the presently evaluated elastogenic compounds, LNA-29a is the most potent stimulator of tropoelastin protein synthesis, especially in adult porcine venous VSMCs. Therefore, the incorporation of LNA-29a in tissue engineering strategies could be a valuable approach to improve the elasticity of the engineered blood vessels.

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Author Disclosure Statement

All the authors declared no competing interests.

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