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Lysyl oxidase interactions with transforming growth factor-β during angiogenesis are mediated by endothelin 1

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Abstract

Crosstalk between multiple components underlies the formation of mature vessels. Although the players involved in angiogenesis have been identified, mechanisms underlying the crosstalk between them are still unclear. Using the ex vivo aortic ring assay, we set out to dissect the interactions between two key angiogenic signaling pathways, vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ), with members of the lysyl oxidase (LOX) family of matrix modifying enzymes. We find an interplay between VEGF, TGFβ, and the LOXs is essential for the formation of mature vascular smooth muscle cells (vSMC)-coated vessels. RNA sequencing analysis further identified an interaction with the endothelin-1 pathway. Our work implicates endothelin-1 downstream of TGFβ in vascular maturation and demonstrate the complexity of processes involved in generating vSMC-coated vessels.

KEYWORDS

angiogenesis, endothelin, lysyl oxidase, TGF-beta, vascular maturation

1 | INTRODUCTION

Vascular development and maintenance of the arterial wall are complex processes. Signal mediated crosstalk between cell types and the extracellular matrix (ECM) are required to form layers that sheath the endothelial cells (EC) with vascular smooth muscle cells (vSMC) and an ECM scaffold of proteins, such as collagens and elastins, to provide structural integrity and elasticity to the artery. Abnormal interactions between these components can result in arterial weakness or fragility,

Abbreviations: EC, endothelial cells; ECM, extracellular matrix; Ednra/ETA, endothelin receptor type A; Ednrb/ETB, endothelin receptor type B; ET1, endothelin 1; FDR, false discovery rate; HUVEC, Human umbilical vascular endothelial cells; LOX, lysyl oxidase; MOVAS, mouse vascular aorta cells; RNAseq, RNA sequencing; TGFβ, transforming growth factor beta; VEGF, vascular endothelial growth factor; vSMC, vascular smooth muscle cells; βAPN, beta-aminopropionitrile.

manifested as arterial aneurysms or dissections. While many of the players involved in arterial development and maintenance have been identified, the mechanisms involved in the crosstalk between them are still unclear. Angiogenesis, the process of generating new vessels from a pre-existing one, can serve as a good model to study the mechanisms that underlie the interactions within the vasculature.

The angiogenic process begins with modulating a specific area of an existing vessel, created by extracellular (mostly paracrine) signals, most predominantly vascular endothelial growth factor A (VEGFA).[1] VEGF induces changes in EC characteristics from epithelial like into mesenchymal-like cells promoting their migration. The ECs lead the growing sprout through the ECM with the help of repulsive or adhesive molecules forming an endothelial sprout. In the following step, EC proliferation is reduced and recruitment of mural cells (pericytes and vSMC) takes place as part of the vessel's maturation.[2] Both cell-types create direct cell-cell contact with the ECs. This maturation phase is mediated by several factors, including transforming growth factor β1 (TGFβ1), playing a major role.[2]

The activity of paracrine signals such as VEGF and TGFβ has been shown to be affected by multiple extracellular factors.[3,4] A key family of secreted matrix modifying enzymes that have been previously demonstrated to modulate the activity of multiple signaling pathways including VEGF and TGFβ are the lysyl oxidases (LOXs).[5-8] This family of enzymes consists of five members including LOX and LOX-Like (LOXL) 1-4.[8] LOX family members have been implicated in multiple processes including angiogenesis and vascular maintenance. The importance of LOX in arterial homeostasis has been demonstrated in humans and mice carrying mutations in the LOX gene. These individuals are susceptible to thoracic aortic aneurysms and dissections.[9-11] Mouse models of LOX inhibition using the pan-LOXs irreversible inhibitor beta-aminopropionitrile (βAPN)[12] demonstrate similar aortic pathologies further reinforcing the significant roles these enzymes play in the vasculature.

Complicating the dissection of LOX in vascular development and maintenance is the broad expression and somewhat overlapping activities of the other LOXL enzymes, where several members are expressed in ECs, vSMCs, and in fibroblasts coating the large vessels.[8] To gain insight into the interplay between key signaling pathways within the vasculature and examine whether LOX family members play a role in this crosstalk, we employed the ex vivo aortic ring assay in which the distinct layers of the vessel are maintained, vascular sprouts form, mature and can be imaged yet all with the benefit of a cultured and controlled environment. Taking advantage of the aortic ring assay and RNA sequencing analyses from distinct treatments, we find novel roles for LOX family members in vascular maturation. We further identify a novel role for the vasoconstrictor protein endothelin-1 (ET1) in vascular maturation. Our results not only implicate the ET1 cascade in a novel process, but they also highlight another layer of complexity in the crosstalk between distinct cell types and interplay between signaling pathways, opening novel avenues for interventions.

2 | MATERIALS AND METHODS

2.1 | Mice

C57BL/6 mice were obtained from Harlan Inc. All experiments involving live vertebrates conform to the relevant regulatory standards [Technion Institutional Care and Animal Use Committee (IACUC) and national animal welfare laws, guidelines, and policies].

2.2 | Aortic ring assay

Aortic ring assay was performed essentially as previously described.[13] Briefly, 6-week-old female mice were anesthetized with isoflurane and decapitated to avoid aortic damage caused by neck dislocation. Later, mice were disinfected with 70% ethanol before cut open. The rib cage was opened at the sides exposing the entire thoracic cavity. The aorta was gently cut loose from the spine using fine scissors and kept in cold Opti-MEM (Gibco) with 1% Penicillin Streptomycin. Following isolation, the aorta was thoroughly cleaned from fat and vascular branches. Next, under a dissecting microscope, it was cut into 0.5 mm width rings. Following an overnight serum starvation, to equilibrate growth factor responses and create a uniform baseline state, the rings were embedded in collagen matrix and fed with growth medium supplemented with the desired treatment.[13] Prior to growth regression, rings were fixed (using 4% paraformaldehyde) and immunofluorescent staining was performed to examine sprout structure and coverage.

For each experimental condition an average of 10 rings was carried out per experiment. Results demonstrated are summaries of at least three independent distinct experiments summarizing ~30 aortic rings. Quantification of immunostaining results is based on at least five representative rings from three independent experiments.

2.3 | Migration assay

HUVECs were kindly contributed by Gera Neufeld's lab and MOVAS cells were contributed by Itzhak Kehat's lab. HUVEC or MOVAS were seeded at close to 100% confluence in an ImageLock 96-well plate and grown in a 37°C, 5% CO_2 incubator. The HUVEC were grown in M199 supplemented with 20% fetal calf serum (FCS), 1% penstrep, 1%

Q and XF (5 ng/mL). The MOVAS were grown in DMEM (Biological Industries) supplemented with 10% FCS, 1% pen-strep and 1% Q. After an overnight starvation (M199 supplemented with 10% FCS, 1% pen-strep and XF (5 ng/mL) and serum-free DMEM supplemented with 1% pen-strep and 1% Q, respectively) the desired treatments were added. A scratch was done using a WoundMaker tool (Essen BioScience), a 96-pin mechanical device designed to create homogeneous scratch wounds per plate. Images were taken at a 30 minutes interval in the IncuCyte ZOOM incubator.

2.4 | Proliferation assays

SMCs- Vascular smooth muscle cells were counted manually (camera) and 20,000 cells were plated in a 12 multiwell plate dishes (Thermo scientific). Cells were kept in Opti-MEM medium (Gibco), 24 and 48 hours post plating, and were then counted using Beckman Z1 coulter particle counter. ECs- HUVEC were seeded on 24-multiwell plate coated with PBS-GEL at 10,000 cells/well in medium supplemented with 20% serum and allowed to attach for 4-5 hours. Cells were changed to media with 10% serum supplemented with VEGF, VEGF + βAPN or VEGF + βAPN + TGF-β and Negative control (no treatment). Cells were allowed to proliferate for 48 hours. Treatments were replaced on a daily basis. Cell number was determined by Beckman Z1 coulter particle counter. Following similar treatments for 48 hours, cells were fixed and stained using anti-Ki67 to directly monitor proliferating cells. Relative proliferation was measured using the ratio between Ki67 expressing nuclei and number of cells.

2.5 | Immunohistochemistry

Immunohistochemistry of aortic rings was performed essentially as described previously.[13,14] The following antibodies were used: αSMA (1:100; Cell Marque), Ki67 (1:100; Cell Marque) and PECAM (1:50; Dianova).

2.6 | Statistical analyses

Statistical analyses were carried out using GraphPad software and T-test or one-way ANOVA were used. Significance was considered as a minimum p value of $<.05$.

2.7 | RNAseq

RNA Sequencing was performed at the Biomedical Research Core Facilities at the University of Michigan. Libraries were prepared using the NEB Ultra Low RNA kit using 30 ng of input RNA. The samples were sequenced 100 bp paired end on Illumina HiSeq2000. The samples were run over 2 lanes, 12 samples per lane, and produced an average of around 50 million reads per sample. Quality control was performed using FastQC (v0.11.2) and over-represented sequences (adapters) were removed using CUTADAPT tool (v 1.8).

2.8 | Differential expression

Reads were then aligned to mouse reference genome and annotation file (Mus_musculus.GRCm38.73 downloaded from ENSEMBL) using Bowtie2 (v2.2.9). The number of reads per gene was counted using summarizeOverlaps (v1.8.1) in R Studio. Differential expression analysis was performed using DESeq2 (version 1.12.0).

2.9 | Pathway analysis

Reads were aligned to mouse reference genome and annotation file (Mus_musculus.GRCm38.80 downloaded from ENSEMBL) using TopHat (v2.0.13) allowing two mismatches per read with options—very-sensitive and—transcriptome-index. The number of reads per gene was counted using Htseq (0.6.1). Differential expression analysis was performed using DESeq2 for further investigation of function and pathway enrichment using Ingenuity Pathway Analysis (IPA) (Bioinformatics 30:523-30).

3 | RESULTS

Members of the LOX enzyme family are best known as key regulators of collagen and elastin crosslinking and as such play a critical role in vascular development and homeostasis.[11,15] However accumulating work has demonstrated that apart from their classical matrix crosslinking activities they also play a role in multiple aspects of cell behavior by regulating signaling pathways.[5,16]

To dissect LOXs' vascular activities and their interactions with signaling pathways in a controlled environment, yet in a way that will maintain the general structure of the vasculature with its distinct layers, we employed the aortic ring assay.[13] Briefly, aortas were dissected from the mice, cleaned and cut into thin (0.5 mm) rings. Following overnight serum starvation to equilibrate their growth factor responses and create a uniform baseline state, the rings were embedded in collagen matrix and fed with growth medium supplemented with the desired treatment.[13] To address the roles of the LOXs in vascular development, we compared aortic rings that were

supplemented with 30 ng/mL VEGF versus ones that were treated with a similar dose of VEGF yet in which the LOXs' enzymatic activities were inhibited. As multiple LOXs are expressed in the distinct aortic layers (Table S1), to block their activities we took advantage of βAPN, an irreversible wide-range inhibitor of all LOXs enzymatic activities[12] (Figure S1A). Treatment of the aortic rings with βAPN only did not result in any change and these were similar to those that have received no treatment (Figure 1A).

Angiogenic sprouts from the aortic rings were observed already two days after incubation with VEGF. By the third day multiple branched sprouts were visible. These sprouts continued to grow and split, until reaching a peak point, as long as VEGF remained in the growth media (Figure 1C). Immunostaining for CD31/PECAM marking ECs and αsmooth muscle actin (αSMA) marking vSMCs demonstrates that 98% of the angiogenic sprouts (n = 111 sprouts) have matured and were coated with vSMCs (Figure 1B-D'). In striking contrast, following the βAPN-mediated inhibition of LOX family enzymatic activities, angiogenesis was dramatically inhibited and sprouts did not emerge even though VEGF was present in the media (Figure 1E; n = 30 rings). Notably, although multiple vSMCs emigrated from the aortic ring, no endothelial sprouts have formed in the VEGF + βAPN-treated rings (Figure 1F,F').

We next wished to test whether βAPN treatment also affects the already mature vSMC coated sprouts. Toward that end, aortic rings were embedded in a collagen matrix as above and supplemented with VEGF for the duration of one week (Figure S1B). By the end of the week highly branched vSMC coated sprouts have formed. In the following week, the rings were maintained in VEGF supplemented media or in VEGF + βAPN media for an additional week followed by immunostaining analysis (Figure S1B). We find that the endothelial sprouts were mostly maintained in both treatment groups (Figure S1C,D) although those cultured in the presence of βAPN were not as organized as those treated with VEGF alone. Notably, although vSMCs were abundant in the matrix, the coverage of the sprouts was severely compromised in the VEGF + βAPN treatment group where endothelial sprouts lost their vSMC coverage and vSMCs seemed disorganized (Figure S1E,F''). These results raise the possibility

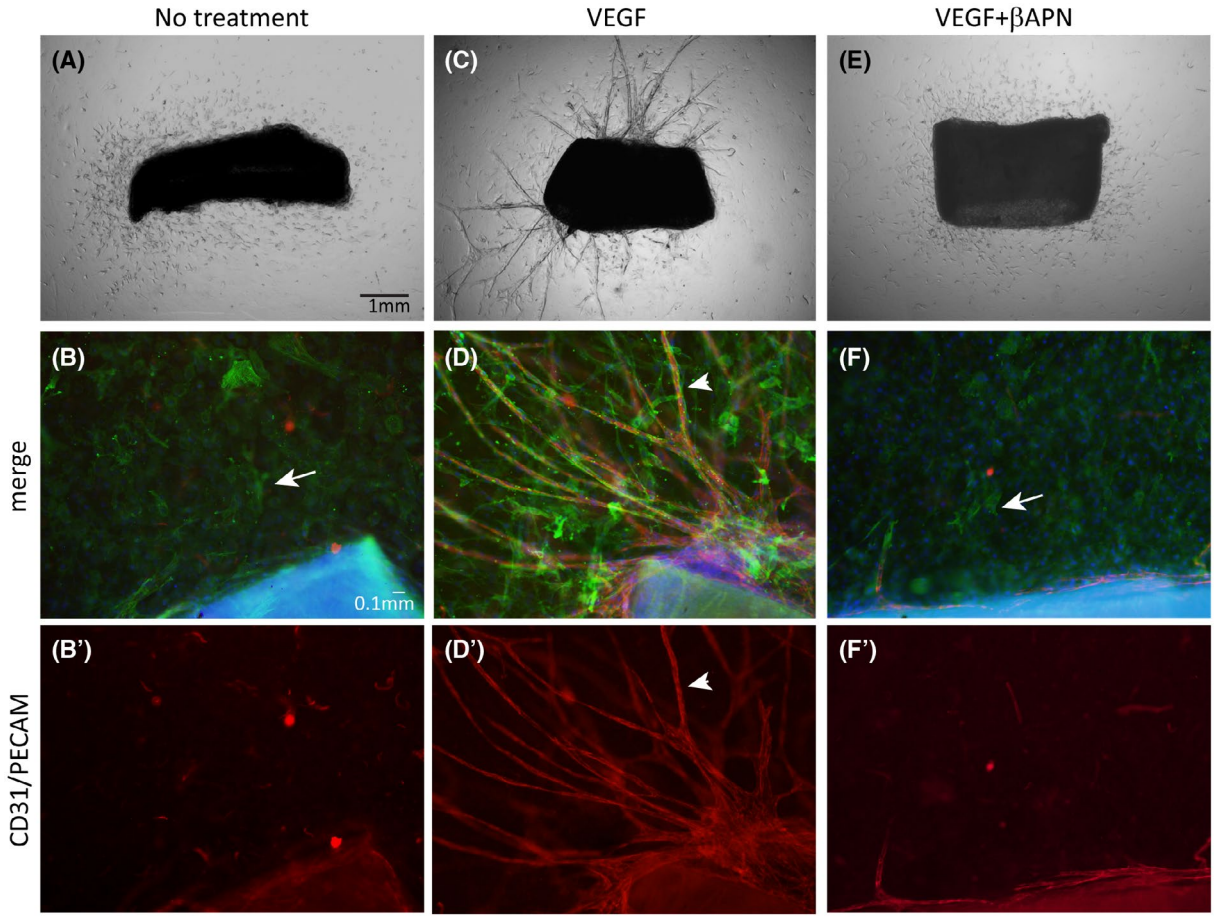

FIGURE 1 βAPN blocks vascular growth. Brightfield and fluorescent immunostaining of aortic rings treated with full medium (A,B,B'), VEGF (C,D,D'), and VEGF + βAPN (E,F,F'). In B, D, and F ECs are marked by CD31/PECAM (red) and vSMCs by αSMA (green). White arrows (B,F) mark vSMCs that have emigrated from the rings yet are not coating nascent endothelial vessels. White arrowhead in marks endothelial sprout (red, D,D') coated with vSMCs (green, D)

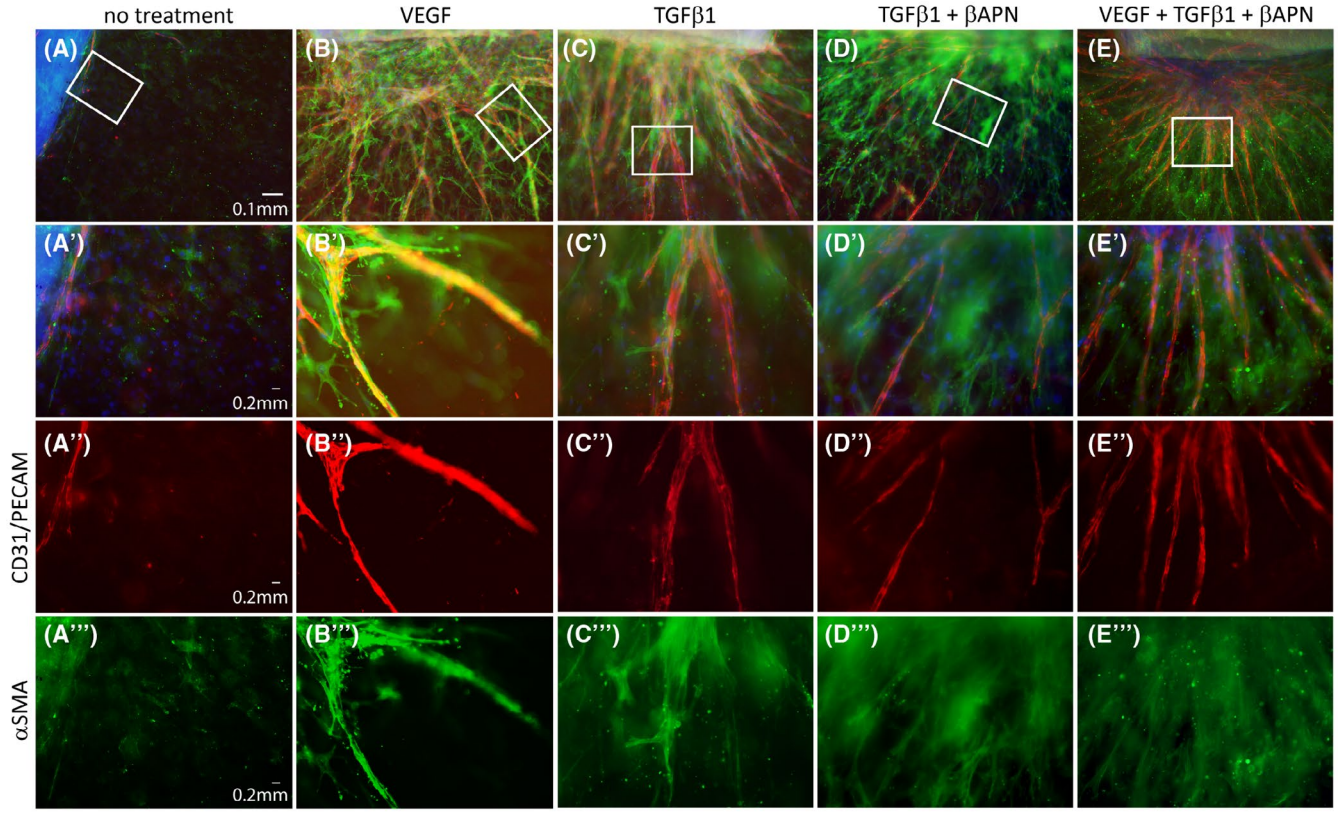

FIGURE 2 TGFβ1 rescues βAPN- mediated inhibition of vessel growth. Immunofluorescent images of aortic rings stained for CD31/PECAM marking ECs (red) and αSMA marking vSMC (green). Treatments are as indicated no treatment (A-A'"); VEGF (B-B'''); TGFβ (C-C'''); TGFβ + βAPN (D-D''') and VEGF + TGFβ + βAPN (E-E'''). Note that although endothelial vessels form following βAPN and TGFβ treatment (D-D'') vSMCs (D''') are disorganized and do not coat the sprouts. In contrast, upon treating the rings with VEGF + TGFβ + βAPN (E-E''') vessels are properly coated by the vSMCs

that blocking LOXs' enzymatic activity affected the interactions between the vSMC and the underlying endothelial cells.

To test what could mediate the crosstalk between the two cell types we searched for a signal that has been implicated in (a) angiogenesis in endothelial cells, (b) vSMC-dependent processes and (c) that is known to interact with LOXs. One such candidate fulfilling the above criteria is the TGFβ pathway. To confirm previous observations and test whether the aortic ring assay could also be used in monitoring TGFβ angiogenic properties, we treated aortic rings with TGFβ1. While no sprouts form in the non-treated rings and numerous vSMC-coated sprouts form in the VEGF-treated rings, we find that following this treatment multiple endothelial sprouts form and that 50% of them (n = 99 sprouts) were coated with vSMC (Figure 2A-C'''). This activity is completely blocked by the addition of a TGFβ Receptor 1 (TGFβR1) kinase inhibitor (Figure S2A,B) demonstrating the specificity of the treatment.

To test whether the inhibition of LOXs enzymatic activity attenuates TGFβ angiogenic activities we cultured the aortic rings with recombinant TGFβ1 and βAPN. We find that although the inhibition was not as robust as observed in the VEGF + βAPN treated rings, a marked reduction in the number of endothelial sprouts that sprouted from the rings as well as in the vSMC coated sprouts was observed although significant vSMCs were spread within the collagen matrix (Figure 2D-D''').

The observation that both VEGF and TGFβ1 could induce endothelial sprouts that are coated with vSMCs and that both these signals are affected by LOX inhibition (although to different levels), raised the possibility TGFβ1 signaling is acting downstream of VEGF, the main angiogenic pathway. To test whether this is the case, we treated aortic rings with VEGF and the TGFβR1 kinase inhibitor and monitored sprout formation. vSMC coated endothelial sprouts have still formed following this treatment demonstrating that TGFβ1 does not act downstream of VEGF in this assay (Figure S2C,D'').

Having verified the independent requirement of both signaling pathways and their dependence on LOXs' activity we wished to test whether activation of both cascades could rescue the βAPN-mediated angiogenic inhibition. Towards that end, following their embedding, the aortic rings were treated with media containing all three factors, VEGF, TGFβ1, and βAPN. Notably, we find that multiple endothelial sprouts form and 70% of these (n = 147 sprouts) are coated with vSMCs (Figure 2E-E'''). These results suggest that an

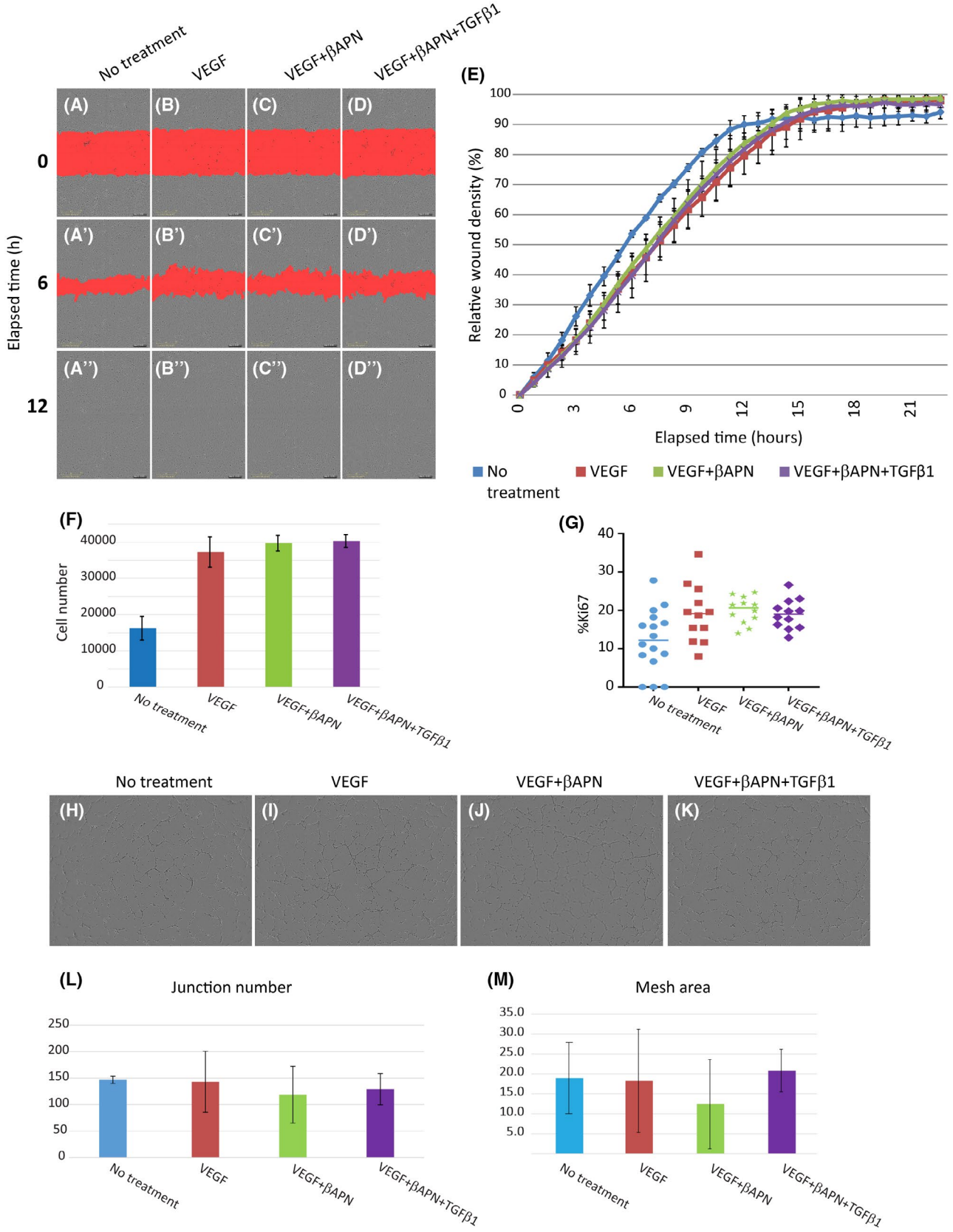

FIGURE 3 βAPN and TGFβ do not affect endothelial migration, proliferation or tube formation. Time course images of HUVECs treated as indicated with the gap lacking cells marked in red (A-D''). Quantification of 'wound' density in the distinct treatments (E). Quantification of HUVEC proliferation (F) and Ki67 immunostaining (G) with the distinct treatments. Images of HUVEC in a cord formation assay with the distinct treatments as indicated (H-K). Quantification of number of junctions (L) and total mesh area (M) demonstrate no significant difference is observed following the distinct treatments

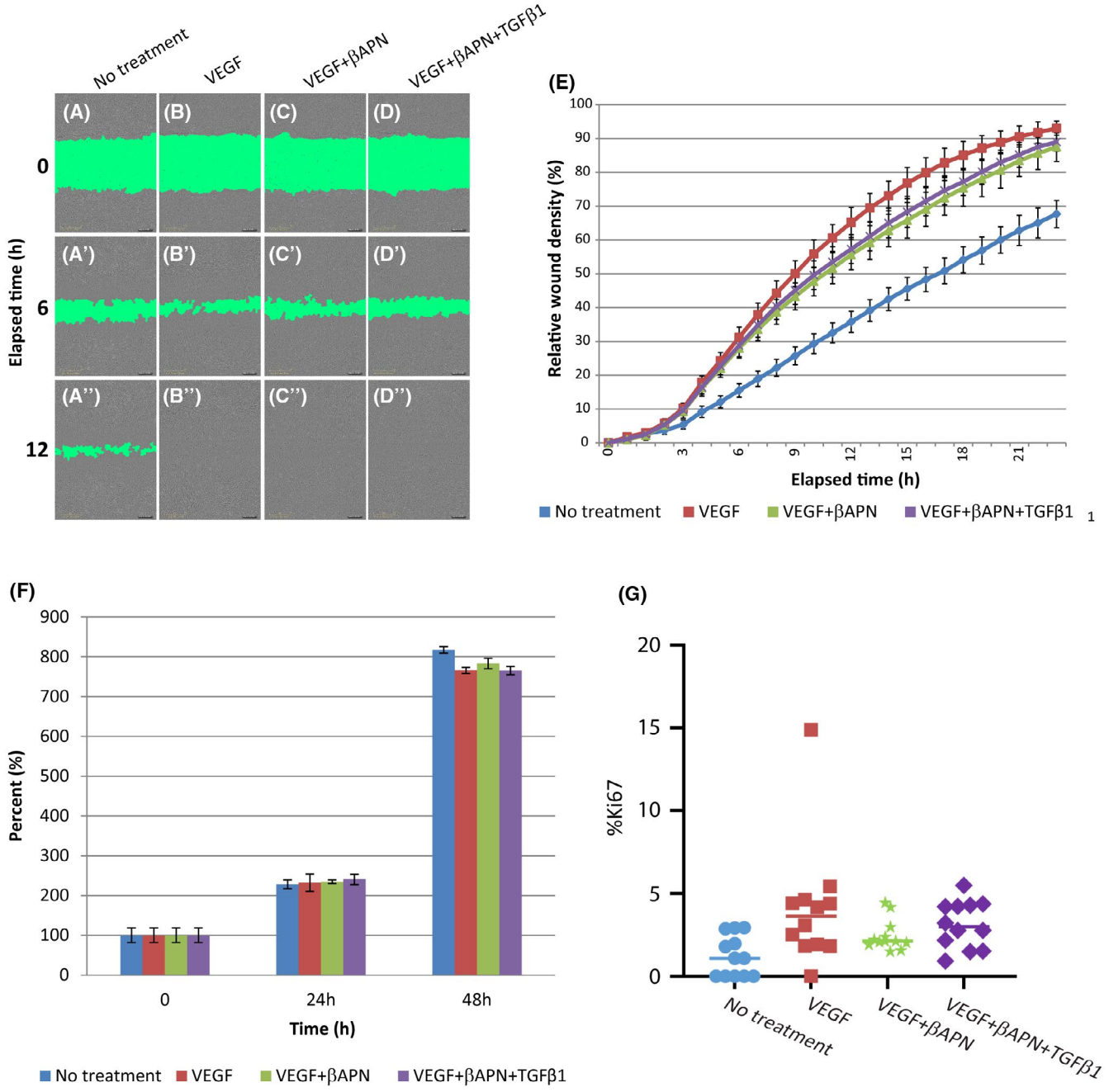

FIGURE 4 βAPN and TGFβ do not affect vSMC migration or proliferation. Time course images of MOVAS treated as indicated with the gap lacking cells marked in green (A-D''). Quantification of "wound" density in the distinct treatments (E). Proliferation assay (F) and Ki67 immunostaining (G) of MOVAS cells treated as mentioned demonstrate no difference following the distinct treatments

interplay between the two pathways could, to a large extent, bypass the dependence on LOXs activity facilitating the formation of mature vSMC-coated endothelial sprouts.

The observation that activation of the two pathways was able to rescue the βAPN mediated angiogenic inhibition could be caused by the cascades' activity on one cell type or on the crosstalk between them. vSMC coating of endothelial sprouts involves multiple processes including cell proliferation, migration, adhesion, and organization (eg, tube/cord formation). Any of these processes could, in principle, be affected by βAPN. To distinguish between these possibilities, we set to treat each cell type independently and monitor the response to the distinct treatments.

Human umbilical vascular endothelial cells (HUVEC) migrate and form cords when grown on matrigel. We thus tested the effects of βAPN and TGFβ1/VEGF on HUVEC migration, proliferation, and cord formation. We find neither LOXs' enzymatic inhibition nor does TGFβ supplementation affect HUVEC migration (Figure 3A-E) or cell proliferation (Figure 3F-G and Figure S3). Likewise these treatments

had no effect on HUVECs cord formation capabilities (Figure 3H-M).

That βAPN did not affect EC behavior (migration, proliferation, and cord formation) raised the possibility that the dramatic angiogenic inhibition observed in the aortic ring assay might be carried out primarily on the vSMCs. Using MOVAS cells, a vSMC line derived from mouse aorta, we set to test whether βAPN and TGFβ affected vSMC behavior. We thus monitored their proliferation, adhesion, and migration, critical properties required for coating the nascent sprouts. In a similar manner to the observed lack of effect on HUVEC, we did not detect major changes in these properties following βAPN or TGFβ administration to the vSMC media (Figure 4 and Figure S4). Altogether, the above results reinforce the notion that the effects mediated by βAPN on the sprouts which are rescued by TGFβ1 are not due to the specific LOXs inhibition in one cell type but possibly on the interplay between them.

To identify how TGFβ1 overcomes the angiogenic inhibition mediated by βAPN, we carried out a RNAseq analysis from aortic rings derived from the following four conditions: (a) no treatment (rings were grown in DMEM only) (b) VEGF; (c) VEGF + βAPN and (d) VEGF + βAPN + TGFβ1. Briefly, the aortic rings were incubated for 3 days with the distinct media (as in Figures 1 and 2) and then RNA was extracted for further analysis. We then ran two group comparisons using the DESeq package in R studio[17] to observe differentially expressed genes within each condition in an unbiased manner. Wald significance tests were run between the untreated and treated groups for each of the three conditions and p-values were corrected using the Benjamini–Hochberg procedure. The largest treatment effect was observed in the VEGF + βAPN + TGFβ1, with 1787 differentially expressed genes (FDR < 0.1). The effect of VEGF alone was significant for 393 genes, while the effect of VEGF + βAPN was only slightly larger with 552 genes showing differential expression (FDR < 0.1).

Among the top five genes in both the VEGF and VEGF + βAPN differentially expressed gene lists was endothelin receptor type B (Ednrb/ETB) (Figure 5, Table S2). Likewise, we found that endothelin receptor type A (Ednra/ETA) showed significantly higher expression in the VEGF treatment group (Figure 5). To observe whether this effect on Ednrb/ETB persisted after the addition of TGFβ1, we queried all endothelin genes in the VEGF + βAPN + TGFβ1 versus untreated results. The endothelin receptors were found to have similar expression to the untreated group, however the endothelin 1 (Edn1) gene itself showed significantly higher expression with VEGF + βAPN + TGFβ1 treatment (Figure 5). For a complete picture of the different effects between each condition, we also ran two group comparisons between each treatment group. Notably, only one gene showed differential expression between the VEGF and VEGF + βAPN conditions at FDR < 0.1 (Table S2).

Should the endothelin cascade be at the core of the crosstalk unveiled by the TGFβ dependent rescue of the vascular sprouts

(A)

	Fold Change			Q-value		
Gene	VEGF	VEGF+βAPN	VEGF+βAPN+TGFβ1	VEGF	VEGF+βAPN	VEGF+βAPN+TGFβ1
Edn1	ns	ns	4.55	ns	ns	8.01E-03
Ednrb	4.16	4.67	ns	1.26E-08	1.39E-10	ns
Ednra	2.33	ns	ns	1.46E-02	ns	ns

(B)

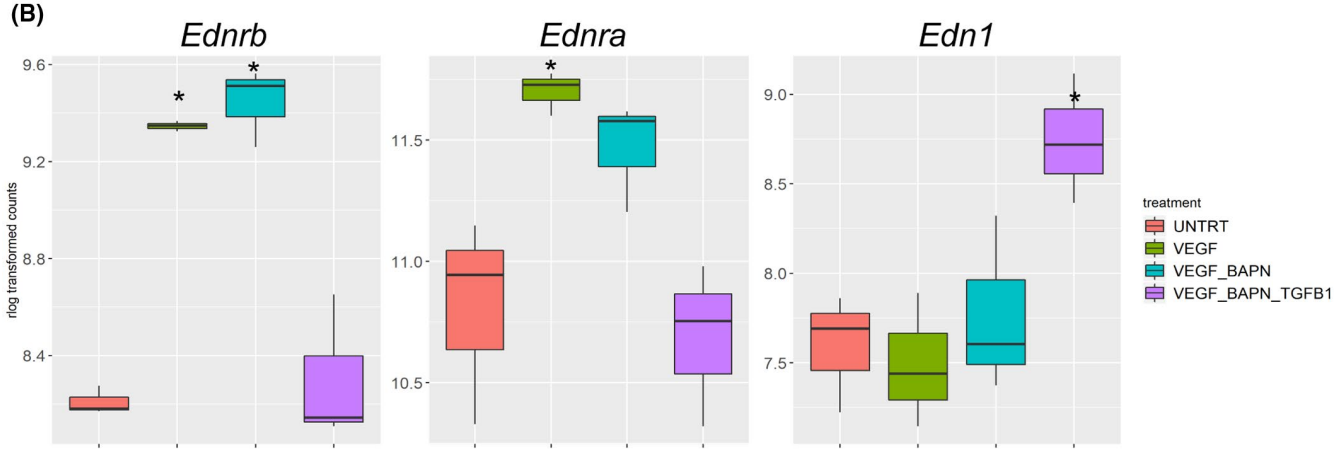

FIGURE 5 Endothelin 1 related gene expression is affected by VEGF, TGFβ, and Lox inhibition. Expression of Edn1, Ednra and Ednrb following the distinct treatments (A, B)

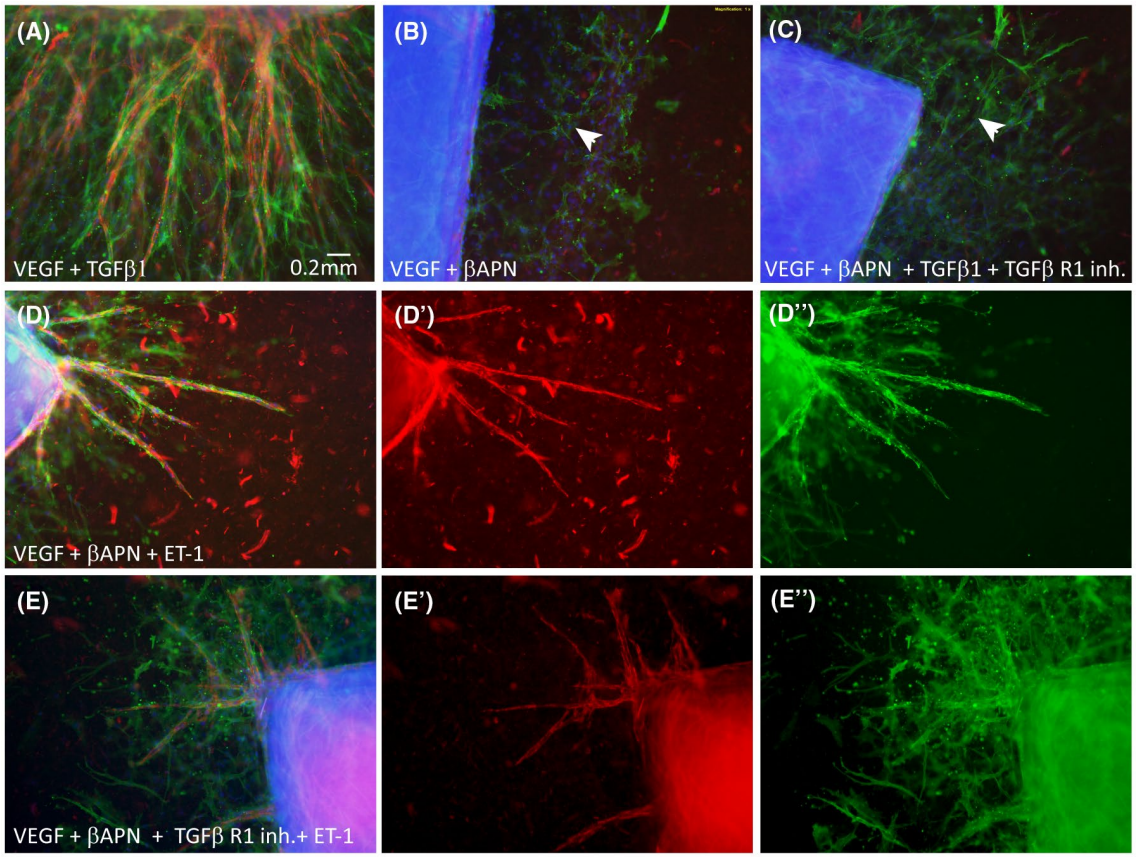

FIGURE 6 ET-1 rescues βAPN-mediated angiogenic inhibition. Immunostaining of aortic rings treated as indicated. Note that although TGFβ R1 inhibitor blocks VEGF mediated angiogenic rescue, it does not affect ET-1 rescue demonstrating ET-1 acts downstream of TGFβ1. Endothelial sprouts are marked by CD31/PECAM staining (red) while vSMC are stained with αSMA (green). White arrowheads (B,C) point to vSMC that have emigrated, demonstrating ring viability, although no sprouts are generated

then activating it also in the presence of βAPN but without supplementing TGFβ1 should induce vascular sprouts. However, activation of the endothelin cascade can also induce TGFβ itself,[18] raising the possibility that such an exogenous induction of the pathway will induce TGFβ which in turn will promote vascular sprouts. To test whether this is the case, or whether recombinant ET-1 (rET-1) can induce sprout formation independently of TGFβ signaling we set to block the latter and assess whether vascular sprouts still form upon rET-1 administration. As a negative control, to test the validity of the inhibition regime, we first cultured the aortic rings in VEGF + βAPN + TGFβ + TGFβR1 inhibitor. As expected, no sprouts form upon this combined treatment and they resembled those cultured in VEGF + βAPN (Figure 6A-C cf. Figure 1E-F').

Treatment of aortic rings only with rET-1 does not induce vascular sprouts, resembling the negative control rings (Figure S5). In contrast, the addition of rET-1 together with VEGF and βAPN promotes mature vSMC coated sprouts (Figure 6D-D''; 21 out of 21 sprouts). To directly test whether this induction is due to ET-1's induction of the TGFβ cascade, we further supplemented the aortic rings' growth media also with a TGFβR1 inhibitor. We find that even upon the inhibition of the TGFβ cascade, vSMC coated sprouts form (Figure 6E-E'''). Altogether, these results may suggest that the activation of the endothelin pathway is acting downstream of TGFβ signaling and able to induce angiogenesis in concert with VEGF and mediate vSMC coating of nascent endothelial vessels.

4 | DISCUSSION

Angiogenic growth is a well-balanced orchestra of events. Proper vessel growth requires synchronization and regulation of multiple events such as endothelial emergence out of the original vessel's wall, proliferation, migration, adhesion, and finally recruitment of vSMC as part of the maturation process. Although many studies have explored these events thoroughly, our understanding is still incomplete. In this work we demonstrated a new interplay between key regulators of the angiogenic process, VEGF and TGFβ1 and the LOX family of enzymes during angiogenesis. We demonstrate the involvement of an additional player, Endothelin-1, as part of the interplay between these factors in this process.

Vascular deformities in lathyric animals[19,20] knockout in mice[8,11,21] and mutations in humans[9,10,22] demonstrated the LOXs are indispensable enzymes during vessel growth and

maintenance. While much focus has been given to their role in the ECM, this activity is usually a matter of a relatively prolonged period of time, even when explored in in vitro studies.[23,24] Here we show, that following a short period of time (3-4 days), a significant phenotype is already observed following these enzymes' inhibition, where regardless of VEGF, vascular sprouting was rapidly inhibited suggesting it may be an ECM unrelated process. Along these lines, accumulating evidence demonstrates that members of the family entail multiple ECM-independent activities. One such example is the oxidation of Platelet Derived Growth Factor Receptor β resulting in *VEGF* mRNA upregulation.[5] Our RNAseq analyses demonstrated that in the βAPN + VEGF treated aortas only few genes' expression was significantly changed in comparison to those treated with VEGF alone therefore suggesting that βAPN did not inhibit the cascade but rather a downstream process. Thus, the observations that LOXs inhibition was sufficient to inhibit VEGF mediated angiogenesis demonstrates these enzymes can also act downstream of VEGF (Figure 1) establishes a new, as yet undemonstrated role for LOXs in regulating vessel growth.

We and others find intertwined interactions between LOX and TGFβ in multiple settings. As a leading contributor to ECM organization, LOX is indirectly responsible for the maintenance of latent TGFβ bound to ECM components. Furthermore, LOX was shown to physically bind TGFβ1 in an enzymatic-independent manner and attenuate its signaling capacity via TGFβ1 oxidation.[25] We have previously demonstrated the interactions between the two also in embryonic muscle development where augmented TGFβ signaling was observed in *Lox* mutant embryos and many of the mutant-associated muscle phenotypes could be rescued by TGFβ inhibition.[6] Interestingly however, when the *Lox* mutant "TGFβ-inhibited" embryos were harvested, numerous hemorrhages were observed at earlier stages and in larger quantities than normally observed in these mutant embryos.[6] These observations suggested that TGFβ signaling might have a protective role in angiogenesis in situations where Lox is missing. Correspondingly, our aortic ring results further reinforce opposite effects of TGFβ1 and LOX (Figure 2). That TGFβ1 rescued the vessels even in the presence of βAPN and that blocking its activity did not affect VEGF signaling, place TGFβ1 downstream to Lox and in parallel to VEGF signaling (Figure 6).

The observation that βAPN treatment had an effect on vSMC coating of the endothelial sprouts in the aortic ring assay but not when administered to the single cell types suggested LOX inhibition affects a crosstalk between ECs and vSMCs. To identify the underlying players, RNAseq analysis was carried out monitoring gene expression changes in the distinct treatments. Surprisingly expression of only a few genes was significantly altered between the VEGF vs. VEGF + βAPN treatments, suggesting the VEGF pathway was not blocked in the βAPN treatment. Along these lines, no effect on cell death was observed suggesting the lack of vascular sprouts was not a result of this process. To facilitate identifying the process at the heart of the phenotypes we searched for an "on-off-on" or "off-on-off" behavior, representing the aortic ring assay phenotypes; VEGF-induced growth, βAPN-mediated inhibition and TGFβ1-mediated rescue. The Endothelin-1 pathway was one such candidate; VEGF induced expression of the ETA and ETB receptors expressed on ECs and vSMCs while TGFβ induced expression of the ligand, ET-1.

Our RNAseq results indicate an association between the ET-1 cascade and the "on-off-on" phenotype detected in the aortic ring assay. In our assays both VEGF and TGFβ1 led to vessel growth, while βAPN-mediated inhibition, drove vessel restriction and inhibited maturation (Figures 1 and 2). Interestingly, in both VEGF and TGFβ1 treatments, where vessel growth prospers, the resulting effect is an activation of the ET-1 pathway, via receptor's upregulation in the VEGF treatment or ligand upregulation in the TGFβ1 treatment (Figure 5). The interplay between TGFβ1 and ET-1 is well documented in the literature. TGFβ1 was shown to induce ET-1 expression in human dermal fibroblasts,[18] matching our RNAseq results where TGFβ1 upregulated ET-1 expression in EC. Moreover, several studies explored TGFβ1 fibrotic effect through the induction of profibrotic gene expression and found it to be ET-1-dependent.[18,26] Thus, it remains possible that TGFβ1-mediated angiogenesis rescue is also ET-1-dependent. Using Bosentan, an antagonist of ET-1 able to bind both ETA and ETB, we aimed to address this question; however, we were not successful as its administration led to a complete arrest of cell emigration (data not shown) suggesting the aortic ring assay may not be a suitable assay for addressing its functions. Further experiments beyond the scope of this work are required to test whether ET-1 is indispensable for the TGFβ1-mediated recovery of growth.

Overall, our results highlight an as yet unexplored role of ET-1 in angiogenesis–vascular maturation. Using the aortic ring assay, we were able to uncover an interaction between the LOXs and the Endothelin pathway that is mediated by TGFβ. Both LOX and ET-1 are known to play key roles in angiogenesis and they both have well-documented influence on ECM, fibrosis, and cancer. Thus, further dissection of their interactions can yield possible new treatment modalities to affect angiogenic pathways.

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DISCLOSURES

The authors confirm no conflict of interests.

AUTHOR CONTRIBUTIONS

H. Grunwald conducted the majority of the experiments. K.L. Hunker and I. Birt performed the RNAseq experiments and analyzed the data. S. Zaffryar-Eilot and R. Aviram assisted in cell culture and Lox activity assays. P. Hasson planned the study, supervised and analyzed the data. S.K. Ganesh and P. Hasson wrote the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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