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## Cell autonomous ANTXR1-mediated regulation of extracellular matrix components in primary fibroblasts

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### Abstract

Our previous studies of *Antxr1* knockout mice suggested that fibrotic skin abnormalities in these mice are associated with increased VEGF signaling. Here, based on studies of primary fibroblasts isolated from skin of *Antxr1*<sup>+/+</sup> and *Antxr1*<sup>-/-</sup> mice at embryonic stage E17.5 and postnatal day P49, we conclude that increased *Colla1* and *Fn1* expression in *Antxr1*-deficient fibroblasts is partly mediated by a cell-autonomous ANTXR1-dependent mechanism. In turn, this may act in parallel with VEGF-dependent regulation of collagen type I and fibronectin production. We demonstrate that *shRNA* mediated knockdown of VEGF in *Antxr1*<sup>-/-</sup> fibroblasts reduces *Colla1* and *Fn1* expression to below control levels, and these are restored by exogenous addition of recombinant VEGF. In addition, the increase in protein levels of collagen type I and fibronectin in mutant cells is blocked by VEGF neutralizing antibody. However, expressing the longest isoform of ANTXR1 (sv1) in mutant fibroblasts decreases levels of *Ctgf*, *Colla1* and *Fn1* transcripts, but has no effect on VEGF expression. Taken together, our data suggest that the increased matrix production in *Antxr1*-deficient fibroblasts primarily occurs via a CTGF-dependent pathway and that other ANTXR1-associated mechanisms contribute to VEGF-dependent increase of collagen type I and fibronectin expression. Our findings provide a basis for further studies of novel ANTXR1-dependent connective tissue homeostatic control mechanisms in healthy individuals, patients with organ fibrosis, and patients with GAPO syndrome.

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#### AUTHOR CONTRIBUTIONS

T.Y.B. and K.H. designed project. K.H. and T.Y.B. performed experiments, collected data and analyzed the data. K.H., B.R.O. and T.Y.B. wrote the paper. All authors read, contributed to discussion and approved the final manuscript.

#### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## Keywords

Extracellular matrix accumulation; skin fibrosis; fibroblasts; ANTXR1/TEM8 GAPO syndrome; VEGF; CTGF; Collagen type I; infantile hemangioma

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## INTRODUCTION

Previous studies showed that the cell surface receptor ANTXR1/TEM8 has a role in adhesion, spreading and migration of cells through its interaction with extracellular matrix (ECM) components [1]. More recently, it was found that loss-of-function mutations in ANTXR1 cause the GAPO syndrome, a recessive disorder with several connective tissue manifestations and vascular anomalies [2]. GAPO patients have significantly reduced life span and die by the third or fourth decade of life; widespread skin and interstitial fibrosis and atherosclerotic changes have been proposed to be the ultimate causes of death [3, 4]. Our previous studies of *Antxr1* knockout mice suggested that fibrotic skin abnormalities in the syndrome are associated with increased VEGF signaling [5].

The anthrax toxin receptor 1, encoded by the *ANTXR1* gene, is a type I transmembrane protein that belongs to the ATR family of receptors [6]. The receptor was initially discovered as a product of tumor endothelium [7]. Similar to members of the integrin family of receptors, ANTXR1 binds to extracellular matrix (ECM) components, such as collagen type VI and I and interacts with cytoskeletal proteins [1, 8, 9].

In addition to high expression in tumor angiogenesis [7, 8, 10–12], ANTXR1 is ubiquitously and broadly expressed in various tissues of endothelial and epithelial origin under normal conditions [13]. Homozygous ANTXR1 loss-of-function mutations cause growth retardation, alopecia, pseudo-anodontia and optic atrophy in GAPO patients [2, 14]. Additional GAPO features include prominent scalp veins, hemangioma-like vascular anomalies, and progressive skin fibrosis [3, 15]. The phenotype of *Antxr1*-deficient mice (*Antxr1*<sup>-/-</sup>) resembles that of GAPO patients [5]. We previously showed that progressive skin fibrosis of *Antxr1*<sup>-/-</sup> mice is associated with accumulation of extracellular matrix components and leaky blood vessels [5]. In addition, similar to what occurs in hemangioma endothelial cells, cutaneous *Antxr1*<sup>-/-</sup> endothelial cells exhibit elevated levels of VEGF-A and activation of VEGFR2 signaling [5, 16].

Given published data indicating that VEGF can induce collagen and fibronectin synthesis in mesangial cells [17, 18] and aggravate fibrosis in models of systemic fibrosis [19], our previously published results raise the question of whether increased matrix protein synthesis in ANTXR1-deficient mice is the result of elevated levels of VEGF in fibroblastic cells. To address this question, in addition to experiments with re-expression of the full-length ANTXR1 splice variant 1 in mutant cells, we treated skin fibroblasts, isolated from 7 weeks-old *Antxr1*<sup>+/+</sup> and *Antxr1*<sup>-/-</sup> mice, with *Vegf*-specific *shRNA* to knock down expression of *Vegf*. Our data reveal an indispensable cell autonomous negative role for ANTXR1 splice variant 1 in regulating production of collagen type I and fibronectin via CTGF. These findings, combined with increased activity of Hif1- $\alpha$  and VEGFR2 in ANTXR1-deficient

fibroblasts, suggest that other ANTXR1-associated mediators may contribute to VEGF-dependent regulation of collagen type I and fibronectin.

## RESULTS AND DISCUSSION

### Antxr1-deficient fibroblasts show increased production of extracellular matrix proteins

The progressive nature of fibrosis in *Antxr1*<sup>-/-</sup> mice suggests that increased collagen production *in vivo* may, in part, be due to postnatal changes and not necessarily direct effects of loss of ANTXR1 in fibroblasts [5]. In fact, increased numbers of macrophages and mast cells in soft tissues, combined with increased levels of cytokines and growth factors, are consistent with this possibility [5]. Nevertheless, fibroblasts isolated from embryonic (E17.5) *Antxr1*<sup>-/-</sup> mice exhibit a significant increase in <sup>14</sup>C-proline incorporation into 30% ammonium sulfate collagen precipitates [5]. Here, by immunoblotting we confirmed the expression of ANTXR1 in fibroblasts of *Antxr1*<sup>+/+</sup> mice (Fig. 1A). Next, we checked whether the dysregulated production of ECM proteins, seen in skin of P49 *Antxr1* null mice, also occurs in *Antxr1* deficient fibroblasts [5]. Transcript levels for *Colla1* and *Fibronectin* (*Fn1*) are slightly, but significantly, higher in embryonic *Antxr1* null fibroblasts at E17.5 (Fig. 1B). Larger differences for *Colla1* and *Fn1* (3.3- and 2.7-fold increase in mutants, respectively) were found in primary fibroblasts isolated from P49 mutant animals. At P49, an up to 50% increase in *Ctgf* transcript levels was also observed in *Antxr1*<sup>-/-</sup> fibroblasts (Fig. 1C). As we show below, these changes in transcript levels are associated with up-regulated collagen type I and fibronectin protein levels in primary fibroblasts of P49 *Antxr1*<sup>-/-</sup> mice (Fig. 4B). By quantifying immunofluorescence data we were able to demonstrate that the % of areas (normalized to 100 cells) stained with antibodies against collagen  $\alpha$ 1(I) and Fn1 is increased in *ko* fibroblasts ~ 5 fold- and ~2 fold, respectively, compared to *wt* controls (Fig. 4B). Levels of staining for collagen  $\alpha$ 1(I) are increased from  $3 \pm 1$  % in control fibroblasts to  $14 \pm 2$  % in knockouts. Similar results were seen for Fn1, with levels increasing from  $6 \pm 2$  % in control fibroblasts to  $12 \pm 1$  % in mutants (mean  $\pm$  s.d.; Fig. 4B). These data suggest that ANTXR1 may act as a negative regulator of fibroblastic synthesis of collagen and other matrix proteins in a cell autonomous manner.

### Increased VEGF signaling is associated with loss of ANTXR1 in knockout mice

Levels of VEGF in plasma and in skin lysates of *Antxr1*<sup>-/-</sup> mice are substantially elevated [5]. High levels of VEGF were shown to aggravate fibrosis in experimental animal models and stimulate collagen production by cultured fibroblasts isolated from the skin in patients with systemic sclerosis [19–22]. To ascertain potential signaling pathways involved in cell autonomous mechanisms of *Antxr1*-dependent regulation of ECM we asked whether levels of VEGF in fibroblasts are affected by loss of ANTXR1. Total protein and RNA lysates were collected from primary fibroblasts isolated from skin of control and mutant animals at embryonic stage E17.5 and postnatal day P49. The levels of *Vegf-a* were different between genotypes at P49 (Fig. 2A); a 3-fold increase of *Vegf-a* expression in mutant cells is consistent with the progressive nature of fibrosis seen in *Antxr1* knockout mice [5]. Protein levels assessed by ELISA showed that VEGF levels in fibroblasts of *Antxr1*<sup>-/-</sup> mice,  $0.8 \pm 0.1$  pg/ $\mu$ g, in mutants were about 40 % higher than in cells isolated from controls,  $0.5 \pm 0.1$  pg/ $\mu$ g (Fig. 2B). Immunoblotting with antibody against VEGF corroborated these data

(Fig. 2C). Moreover, increased *Vegfr2* transcript levels and phosphorylation of VEGFR2, the key transducer of VEGF signaling, indicated an augmented activation of VEGF signaling in *Antxr1*-deficient fibroblasts (Fig. 2C).

Data from previous studies of infantile hemangioma suggested that an ANTXR1/TEM8 containing protein complex regulates  $\beta 1$  integrin-dependent adhesion of endothelial cells to fibronectin and collagen I [16]. In addition, control of VEGF-dependent signaling in vascular endothelial cells is critically dependent on interactions between  $\beta 1$  integrin, VEGFR2 and ANTXR1/TEM8 [16]. Moreover, ANTXR1/TEM8 appears to play an essential role in suppressing VEGF signaling in endothelial cells [16]. Similar to hemangioma endothelial cells, endothelial cells isolated from *Antxr1* null mice exhibit abnormal activation of VEGFR2 signaling [5]. Activation of VEGFR2 is also detected in *Antxr1*  $-/-$  fibroblasts (Fig. 2C). Thus, the regulatory mechanism in endothelial cells may also apply to *Antxr1*-deficient fibroblasts. Increased VEGFR2 activation may further promote VEGF expression in a positive feedback manner [23].

### VEGF promotes production of collagen type 1 and fibronectin in fibroblasts

The elevated levels of *Vegf* and *Vegfr2* transcripts, and the high VEGF protein levels and VEGFR2 activities (p-VEGFR2) in *Antxr1*  $-/-$  fibroblasts (Fig. 2) raise the question of whether increased matrix protein synthesis in *Antxr1*-deficient fibroblasts is mediated by VEGF levels. To address this possibility, we used the following approaches. First, we utilized *shRNA*-mediated knockdown of VEGF in fibroblastic cells isolated from the skin of mutant animals. The efficiency of *shVegf*-targeting was evaluated by ELISA assays (Fig. 3A). Two stable cell line clones, *shVegf-1* and *shVegf-2*, demonstrated a significant down regulation of VEGF and were chosen for assessment of transcript levels of ECM components. An 80 % reduction of mRNA levels of *Colla1* and *Fn1* in *shVegf-1* and *-2* lines suggests that VEGF is responsible for their up-regulation in *Antxr1* null fibroblasts (Fig. 3B). This was further confirmed when addition of recombinant mouse VEGF164 (10 ng/ml) resulted in a dramatic increase of *Colla1* and *Fn1* transcript levels in *shControl* and *shVegf*-treated fibroblast cultures (Fig. 3C). Protein levels of collagen  $\alpha 1(I)$  and fibronectin were also more than 2-fold increased after treatment of wild type fibroblasts with 25 ng/ml of recombinant VEGF for 24 hours (Fig. 4A). In contrast, treatment of *Antxr1*  $-/-$  fibroblasts with 1 $\mu$ g/ml of VEGF monoclonal antibody for 48 hours resulted in  $\sim 2$  fold reduction of collagen  $\alpha 1(I)$  and Fn1 levels as assessed by immunofluorescence (Fig. 4B). The area stained with antibodies against collagen  $\alpha 1(I)$  was reduced from  $14 \pm 2$  % in *Antxr1* null fibroblasts to  $8 \pm 2$  % after treatment of cells with VEGF monoclonal antibody. Similar results were seen for Fn1; the level of staining for fibronectin dropped from  $12 \pm 1$  % in knockout fibroblasts to  $7 \pm 2$  % in treated cells (mean  $\pm$  s.d.; Fig. 4B). In contrast, there was no significant elevation of *Ctgf* transcripts after VEGF treatment (Fig. 3C). These data suggest that increased *Ctgf* levels in ANTXR1-deficient cells may occur either by a mechanism that is upstream of VEGF or that CTGF and its regulation of extracellular matrix components is independent of VEGF. Restoration of ANTXR1 levels in *Antxr1* null cells should help to distinguish between these two possibilities.

## ANTXR1 regulates *Ctgf* and fibroblastic synthesis of matrix proteins in a cell-autonomous manner

Alternative splicing of *Antxr1* results in five different splice variants (sv1, sv2, sv3, sv4, sv5) [7, 24]. Previous studies provided evidence for direct interaction between ANTXR1 and the actin cytoskeleton and demonstrated a dramatic effect of ANTXR1 loss on cytoskeletal organization [2, 25]. Moreover, a study by Go and colleagues suggested that cytoskeletal dynamics regulates ANTXR1 function and its association with the extracellular matrix [26].

Thus, to dissect role of VEGF in regulation of ECM components, primary P49 *Antxr1*<sup>-/-</sup> fibroblasts were transiently transfected for 72 hours with pcDNA3-ANTXR1-sv1-HA plasmid expressing the longest and only ANTXR1 isoform containing an actin-binding site. The generation of pcDNA3-ANTXR1-sv1-HA (sv1) plasmid has been described previously [25]. First, by quantifying western blots we confirmed 70% efficiency of sv1 transfection (normalized to  $\beta$ -actin, relative levels of expression of ANTXR1 and sv1 are 1.41 and 0.98, respectively) (Fig. 5A). Next, we used quantitative assays for *Ctgf*, *Colla1*, *Fn1* transcripts and VEGF protein levels. Immunofluorescence for collagen  $\alpha$ 1(I) and fibronectin was carried out for the assessment of protein expression in lysates obtained from P49 of control (*wt*) and *Antxr1* null (*ko*) fibroblasts treated with vehicle or sv1- containing expression plasmid (*ko*/sv1).

As shown in Figure 5B, re-expressing ANTXR1 (sv1) in *Antxr1* null fibroblasts restored *Colla1* and *Fn1* transcripts to *wt* levels. In addition, protein expression of collagen  $\alpha$ 1(I) and fibronectin was dramatically reduced in sv1- transfected cells, compared to control *ko* cells (Fig. 5C). However, re-expression of ANTXR1 sv1 in mutant cells had no effect on transcript and protein levels of VEGF (Fig. 5B and D) and Hif1- $\alpha$  (Supplemental Fig. S1). In contrast, *Ctgf* transcript levels in *ko*/sv1 cells were brought back to the levels of *wt* controls (Fig. 5B). Taken together, these data suggest that CTGF, a target of ANTXR1, primarily contributes to up-regulation of collagen type I and fibronectin in *Antxr1* null P49 fibroblasts (Fig. 5B and E). The data also raise the question of why expression of sv1 in mutant fibroblasts does not restore all the consequences of ANTXR1 deficiency. Further studies are clearly needed to address this question, but a potential explanation may be that other splice variants play a role in regulating specific pathways. For example, in a previous paper [16] we demonstrated that the secreted isoform sv3 interferes with ANTXR1 functions and induces elevated levels of Hif1- $\alpha$  and VEGF signaling in vascular endothelial cells.

VEGF is regulated by many factors, including growth factors, such as FGF2, hormones, transcription factors and mechanical stimuli [27]. Among many stimuli, the hypoxia inducible factor-1 (Hif1- $\alpha$ ) is considered a major driver of VEGF expression [28, 29]. It has been demonstrated that fibroblasts in the papillary layer are sufficiently hypoxic to have elevated levels of Hif1- $\alpha$  and stimulated VEGF expression, and fibroblasts are known to express the Hif1- $\alpha$  target gene *Cxcl12*. In equine dermal fibroblasts, hypoxia has been shown to result in increased fibroblast proliferation and up-regulated *Colla1* expression [30]. In addition, recent evidence indicates that VEGF, VEGFR1 and VEGFR2 can be expressed at relatively high levels in ligament and tendon fibroblasts as well [31]. Previously, we demonstrated that skin lysates from *Antxr1* knockout mice contained elevated levels of Hif1- $\alpha$  [5]. In addition, Hif1- $\alpha$ /FGF2 signaling is enhanced in *Antxr1* null

fibroblasts (Supplemental Fig. S1A). Therefore, elevated Hif1- $\alpha$  levels may contribute to the increased VEGF expression, resulting in up-regulation of ECM components in *Antxr1* deficient fibroblasts. Such regulatory effects of VEGF on collagen production are consistent with previous reports demonstrating a striking inductive effect of VEGF on collagen expression in cultured fibroblasts and in skin fibrosis [19–22]. It was also shown that VEGF induces fibronectin secretion in human airway smooth muscle cells in an ERK1/2 dependent manner [32]. ERK1/2 activity, a major downstream target of VEGF-dependent signaling is activated in skin of *Antxr1* knockout mice [5]. Both CTGF and VEGF are involved in regulation of ECM production and a shift in the balance between CTGF and VEGF is associated with a switch from angiogenesis to fibrosis [19–22, 33]. It has also been reported that overexpression of CTGF has an inhibitory effect on VEGF levels resulting in severe fibrotic scarring and reduced VEGF-dependent angiogenesis [34]. Importantly, the fact that we found transcript levels of *Coll1a1* and *Fn1* to be increased in ANTXR1-deficient E17.5 embryonic fibroblasts, without significant changes in *Ctgf* and *Vegf-a* levels (data not shown), suggests that loss of ANTXR1 also has a CTGF/VEGF-independent effect on expression of collagen I and fibronectin (Fig. 5E). The progressive nature of fibrosis in *Antxr1*<sup>-/-</sup> mice suggests that increased collagen production *in vivo* may, in part, be due to a postnatal shift in balance between CTGF and VEGF [5]. In summary, our data demonstrate that ANTXR1 produced by fibroblasts regulates extracellular matrix production at embryonic stages and postnatally. ANTXR1-dependent effects are may primarily mediated by CTGF and partly through indirect VEGF- dependent mechanisms (Fig. 5E). Ongoing studies will further elucidate ANTXR1-CTGF-VEGF relationships in orchestrating regulation of extracellular matrix component expression by fibroblasts.

Altogether, our data suggest that ANTXR1 signaling in fibroblasts play an important role in the synthesis of matrix proteins. The novel cell autonomous mechanism of ANTXR dependent regulation of ECM production described here, potentially contributes to fibrous connective tissue accumulation during tissue reparative or regenerative processes. Given that loss of ANTXR1 function potentiates CTGF and VEGF up-regulation in human and mouse models [5, 16], it is appealing to speculate that dynamic regulation of CTGF and VEGF may be a part of an anti-fibrotic repair program; when ANTXR1 is absent, fibrosis occurs. The regulatory effect of CTGF and VEGF on ECM deposition may help explain accumulation of ECM and skin fibrosis in GAPO syndrome, and provides a potential mechanism for other syndromes with fibrotic skin abnormalities.

## METHODS

### Mouse line

*Antxr1* null mouse line used in current study was described previously [5]. Knockout mice were initially generated using the C57BL6/NTac strain and subsequently crossed into the C57BL6/J background.

### Isolation of primary mouse embryonic fibroblasts and fibroblasts from adult mice

P49 fibroblasts and fibroblasts from E17.5 embryos were isolated and characterized as described previously [5]. Cells were isolated from *Antxr1*<sup>+/+</sup> and *Antxr1*<sup>-/-</sup> mice (n=3

independent experiments for genotype). Cells were cultured in DMEM medium supplemented with 15% FBS.

### Western blots

The 70–80% confluent cell lysates were collected using M-PER® buffer supplemented with protease and phosphatase inhibitor cocktail (Roche Applied- Science). Protein concentrations were measured by Bradford assay (Thermo Scientific). Lysates containing 10–30 µg of protein were electrophoresed using 10% SDS-bispolyacrylamide gels, and separated proteins in the gels were transferred to nitrocellulose membranes. After 1 hour blocking in 5% milk/TBST, the membranes were incubated overnight at 4 °C with primary antibodies diluted in 4% BSA (Sigma Aldrich)/TBST solution. After washing, the membranes were incubated with secondary antibodies for 1 h at room temperature and immunoreactive bands were visualized by chemiluminescent substrate (Thermo Scientific). Secondary antibodies, anti-mouse HRP (1:1000, #32430, Thermo Scientific), anti-rabbit HRP (1:10,000, #31460, Thermo Scientific) and anti-goat HRP(1:5000, sc-2056, Santa Cruz) were diluted in 5% milk/TBST solution. The following primary antibodies were used for western blotting: ANTXR1 (1:1000, ab21269, Abcam), Flk1 (1:1000, sc-504, Santa Cruz), p-Flk1 (1:1000, sc-101820, Santa Cruz), Flt1 (1:1000, sc-316, Santa Cruz), VEGF-A (1:500, sc-152, Santa Cruz), and β-actin (1:5000, A5441, Sigma Aldrich) and HA (1:500, sc-805, Santa Cruz).

### ELISA assays

VEGF protein levels in cell lysates of fibroblast were assessed using the Quantikine Mouse VEGF- Immunoassays (R&D Systems) in accordance with the manufacturer's instructions; VEGF in lysates from confluent fibroblast cultures were normalized to protein levels. The optical density of each well was determined using a microplate reader set to 450 nm.

### Generation of stable *shVegf* cell lines and ANTXR1-sv1 transiently expressing cells

Cultured fibroblasts from mutant mice were used to generate cells with *Vegf* gene knockdown. The cells were treated with VEGF shRNA Lentiviral Particles (sc-36815-V, Santa Cruz), Control shRNA Lentiviral Particles (sc-108084, SantaCruz), in the presence of 5µg/ml Polybrene (sc-134220, Santa Cruz). To select stable clones expressing shRNA, cells were treated with 2 µg/ml puromycin dihydrochloride (sc-108071, SantaCruz) until resistant colonies were identified and used for subsequent experiments.

The generation of pcDNA3-ANTXR1-sv1-HA plasmid has been described previously [26]. Primary Antxr1-deficient fibroblasts were transfected with pcDNA3-ANTXR1-sv1-HA using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to manufacture instructions. After 72 hours of treatment, fibroblasts expressing pcDNA3-ANTXR1-sv1-HA and control cells treated with vehicle were analyzed (2 independent experiments in triplicates).

### Quantitative real-time PCR

Total RNA was isolated and first strand cDNA was synthesized using iScript (Bio-Rad). Quantitative real-time PCR with a BioRad iCycler® used primers mixed with the

iQ<sup>TM</sup>SYBR®Green Supermix (Bio-Rad). Primer sets for *Col1a1*, *Fn1*, *Ctgf*, *Vegf*, *Flt1*, *Flk1* and *Gapdh* were purchased from Invitrogen. The sequences of forward and reverse primers are given in the supplemental Table 1 [5]. Specificity of reactions was determined by melting curve analysis. The relative fold changes of gene expression between each gene of interest were calculated by standard curve method. For each gene of interest 3 independent experiments were performed and each experiment was run in duplicates or triplicates.

### Immunofluorescence

Fibroblasts isolated from *Antxr1*<sup>+/+</sup> and *Antxr1*<sup>-/-</sup> mice were seeded on culture slides (BD Falcon). Follow 24 hours of serum withdrawal, exogenous recombinant VEGF (25 ng/ml, R&D) was added to near confluent control and mutant fibroblasts cultured in medium containing 0.5% of serum. After 24 hours of treatment cells were stained and subjected to quantification analysis. For inhibition assays, VEGF neutralizing antibody (1µg/ml, AF-493-NA, R&D) was added to near confluent *Antxr1*<sup>-/-</sup> fibroblasts growing in culture medium containing 10% serum. Cells were ready for analysis after 48 hours of incubation. For staining cells were fixed in 4% PFA for 30 min, washed with PBS, blocked with 4% of normal serum and incubated at 4°C overnight in solution containing the following primary antibodies: Collagen α1(I) (1:100, ab21286, Abcam), or fibronectin (1:100, sc-9068, Santa Cruz), followed by Alexa 555, Alexa 594 or Alexa 488 labeled secondary antibody (1:200, Life technologies or 1:500, Thermo Scientific Inc., respectively). The sv1 or vehicle transfected cells were used for the incubation with primary antibodies against collagen α1(I), fibronectin and Hif1-α (1:100, sc-10790, Santa Cruz) after 72 hours of post-transfection. The overnight, at 4°C incubation with primary antibodies followed by staining with Alexa Fluor 488 (1:500, Thermo Scientific Inc.) for 1hr at room temperature. Nuclei were stained with Hoechst 33342 fluorescent dye (Thermo Scientific).

Image J was used for collecting data on the degree of immunofluorescent staining. For each condition, 3–6 fields from duplicate plates were used for analysis, covering a total of 180 to 240 cells. The percentage of areas stained with antibodies against collagen α1(I) or fibronectin, was normalized to 100 cells.

### Statistical analysis

Data were analyzed using unpaired 2-tailed Student's t-test. All results were expressed as mean ± standard deviation or mean ± standard error (data from 3 independent experiments). Results were considered significant at P < 0.05.

### Study approval of animal use

All animal experiments were done according to the basic protocols approved by the Harvard Medical Area Standing Committee and in an agreement with the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



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## Abbreviations

|               |   |
|---------------|---|
| <b>GAPO</b>   | acronym for growth retardation, alopecia, pseudoanodontia and progressive optic atrophy |
| <b>ECM</b>    | extracellular matrix  |
| <b>CTGF</b>   | connective tissue growth factor   |
| <b>VEGF</b>   | vascular endothelial growth factor A  |
| <b>TEM8</b>   | tumor endothelial marker 8  |
| <b>ANTXR1</b> | Anthrax toxin receptor 1  |
| <b>sv1</b>    | splice variant 1  |

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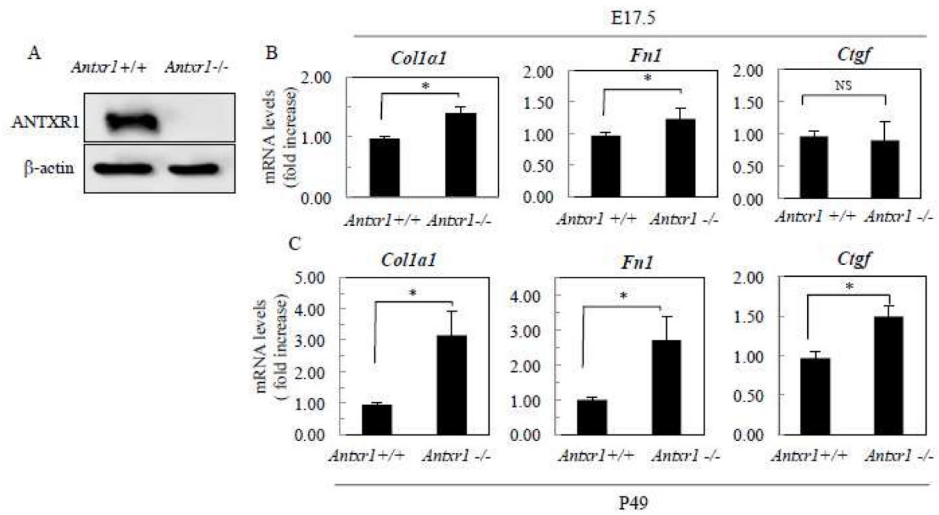
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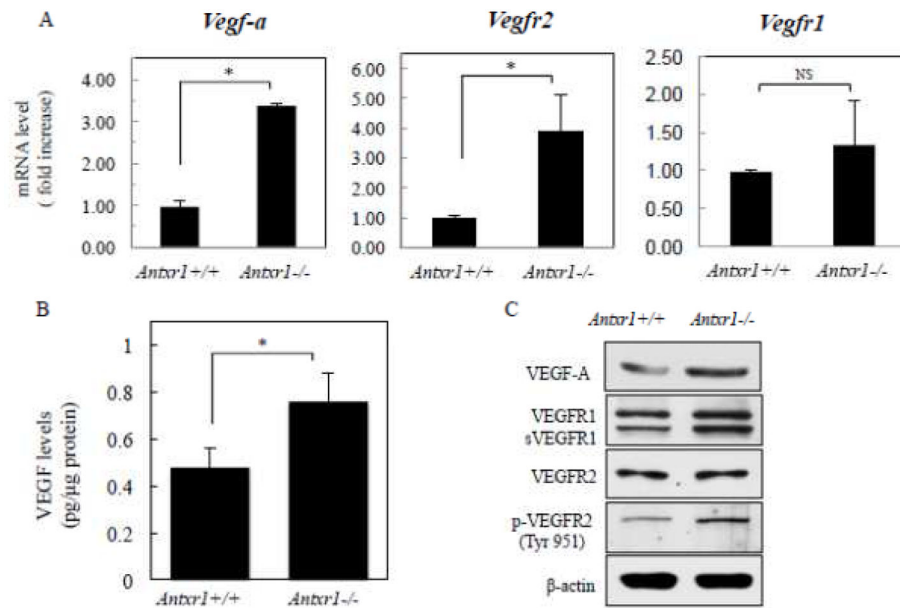
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### Highlights

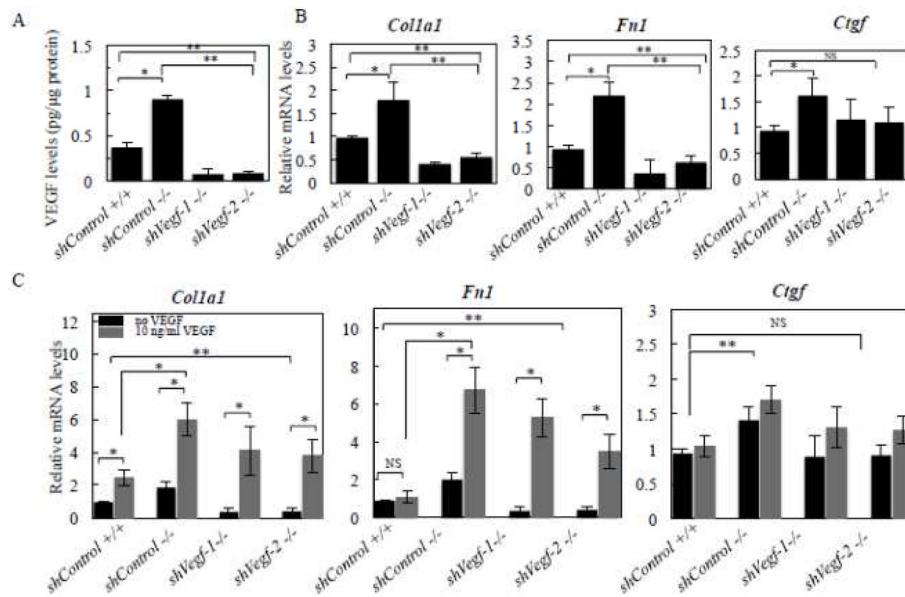
We appreciate the thorough comments provided by the reviewers and the editor and have addressed each of the concerns of the reviewers. In revised version of the manuscript entitled “Cell autonomous ANTXR1- mediated regulation of extracellular matrix components in primary fibroblasts” we took advantage of the availability of a pcDNA3-ANTXR1-sv1-HA (sv1) construct that we obtained from Dr. Mogridge. The results of re-expressing the longest ANTXR1 isoform, containing the actin binding site and implicated in ECM interaction/regulation, in *Antxr1* null fibroblasts provide deeper mechanistic insights into collagen type I and fibronectin regulation. Quantitative measurements of VEGF, CTGF and matrix components in sv1 – transfected and untransfected cells helped to dissect the role of VEGF in ANTXR1-dependent regulation of matrix expression.



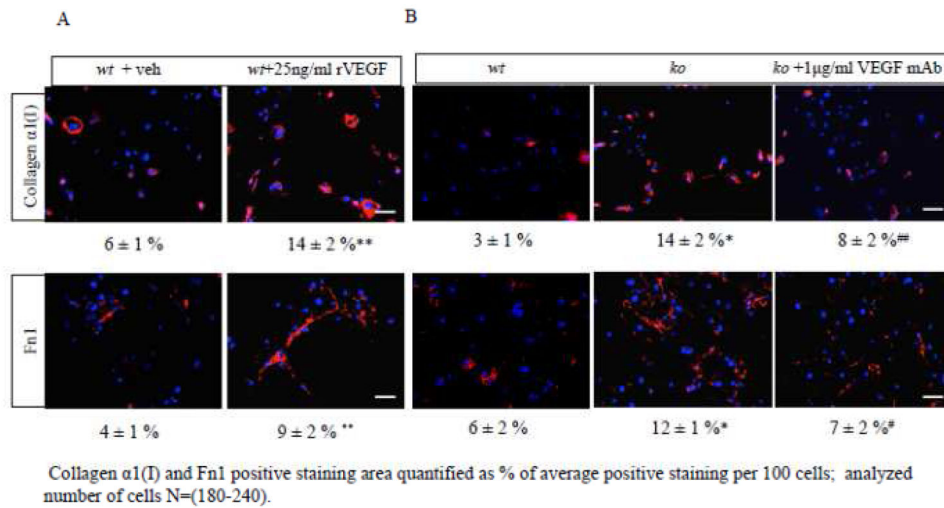
**Fig. 1.** Loss of ANTXR1 is associated with increased transcript levels of ECM components. (A) Western blotting of fibroblasts isolated from *Antxr1*<sup>+/+</sup> and *Antxr1*<sup>-/-</sup> mice at P49. (B, C) Bar graphs show average transcript levels (fold increase) in lysates of control and mutant cells at E17.5 and P49. (\* $P < 0.05$ ,  $n = 6$  per group).



**Fig. 2.** Mutant fibroblasts express high VEGF levels. (A) Bar graphs show average transcript levels (fold increase) in control and mutant fibroblasts isolated from P49 mice. ( $*P < 0.05$ ,  $n = 6$  per group). (B) VEGF protein levels were assessed by ELISA ( $*P < 0.05$ ,  $n = 4$  per group). (C) Immunoblotting of lysates from control and mutant fibroblasts of P49 mice.

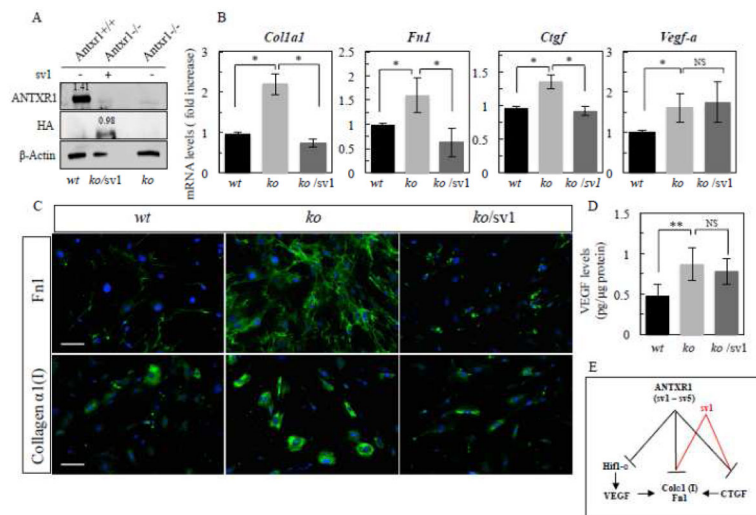


**Fig. 3.** VEGF induced transcript levels of collagen α1(I) and fibronectin. (A) VEGF protein levels were assessed by ELISA ( $*P < 0.05$ ,  $n = 4$  per group) in *shControl* and *shVegf*-treated fibroblasts ( $*P < 0.05$ ,  $n = 4$  per each group). (B) Bar graphs showing average transcript levels (fold increase) of *shControl* and *Vegf* knockdown cell lines ( $*P < 0.05$ ,  $n = 4$  per group). (C) RT-PCR data show transcript levels after treatment of cells with 10 ng/ml of recombinant VEGF (rVEGF). ( $*P < 0.05$ ,  $n = 4$  per group).



**Fig. 4.** VEGF regulates collagen α1(I) and Fn1 protein levels. (A, B) Representative immunofluorescence images and data analyses of cells treated with 1μg/ml of VEGF neutralizing antibody or with 25 ng/ml of rVEGF. (n= 3; \*, #,  $P<0.01$ ; \*\*, ##,  $P<0.05$ ; where \*,\*\* vs. wt (*Antxr1*<sup>+/+</sup>) and #, ## vs. ko (*Antxr1*<sup>-/-</sup>)). Scale bar is 100 μm; veh-vehicle. For each condition, 3–6 fields from duplicate plates were used for analysis, covering a total of 180 to 240 cells. The percentage of areas stained with antibodies against collagen α1(I) or fibronectin, was normalized to 100 cells.





**Fig. 5.** Re-expression of ANTXR1(sv1) restored levels of *Colla1* and *Fn1* transcripts. (A) Assessment of sv1 transfection efficiency by western blotting of lysates from P49 control (*wt*) and *Antxr1* null (*ko*) fibroblasts treated with vehicle or sv1pcDNA3-ANTXR1-sv1-HA (sv1) expression plasmid (*ko/sv1*). Numbers above the bands represent relative expression levels of ANTXR1 and ANTXR1-sv1 proteins normalized to  $\beta$ -actin. (B) Bar graphs showing average transcript levels (fold increase) of *Ctgf*, *Colla1*, *Fn1* and *Vegf-a* ( $**P < 0.01$ ; *ns*-not statistically significant,  $n = 4$  per group). (C) Immunofluorescence staining for collagen  $\alpha 1(I)$  and fibronectin in cultures of fibroblasts from P49 control (*wt*) mice and fibroblasts from *Antxr1* null (*ko*) mice treated with vehicle (*ko*) or sv1- containing expression plasmid (*ko/sv1*). Scale bar is 50  $\mu$ m. Representative images are from triplicates of 2 independent experiments. (D) VEGF protein levels were assessed by ELISA in sv1 transfected and untransfected fibroblasts ( $**P < 0.05$ ; *ns*-not statistically significant,  $n = 4$  per each group). (E) Schematic diagram of ANTXR1-dependent regulation of *Colla1* and *Fn1* in fibroblasts. ANTXR1 (including all 5 splice variants sv1- sv5) negatively modulates *Ctgf*, *Colla1* and *Fn1*, *Vegf-a* and *Hif1- $\alpha$* /expression (black lines) and sv1 negatively modulates *Ctgf*, *Colla1* and *Fn1* only (red lines). ANTXR1-dependent increase of collagen  $\alpha 1(I)$  and fibronectin expression in mutant fibroblasts is primarily mediated by sv1/CTGF-dependent mechanism. Other ANTXR1 splice variants or unknown player (s), downstream of ANTXR1 may contribute to *Hif1- $\alpha$* /VEGF-dependent matrix production in *Antxr1* null fibroblasts.