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Endothelin-1 Mediated Induction of Extracellular Matrix Genes in Strial Marginal Cells Underlies Strial Pathology in Alport Mice

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Abstract

Alport syndrome, a type IV collagen disorder, manifests as glomerular disease associated with hearing loss with thickening of the glomerular and strial capillary basement membranes (SCBMs). We have identified a role for endothelin-1 (ET-1) activation of endothelin A receptors (ET_AR) in glomerular pathogenesis. Here we explore whether ET-1 plays a role in strial pathology. Wild type (WT) and Alport mice were treated with the ET_AR antagonist, sitaxentan. The stria vascularis was analyzed for SCBM thickness and for extracellular matrix (ECM) proteins. Additional WT and Alport mice were exposed to noise or hypoxia and the stria analyzed for hypoxia-related and ECM genes. A strial marginal cell line cultured under hypoxic conditions, or stimulated with ET-1 was analyzed for expression of hypoxia-related and ECM transcripts. Noise exposure resulted in significantly elevated ABR thresholds in Alport mice relative to wild type littermates. Alport stria showed elevated expression of collagen $\alpha 1$ (IV), laminin $\alpha 2$, and laminin $\alpha 5$ proteins relative to WT. SCBM thickening and elevated ECM protein expression was ameliorated by ET_AR blockade. Stria from normoxic Alport mice and hypoxic WT mice showed upregulation of hypoxia-related, ECM, and ET-1 transcripts. Both ET-1 stimulation and hypoxia up-regulated ECM transcripts in cultured marginal cells. We conclude that ET-1 mediated activation of ET_AR on strial marginal cells results in elevated expression of ECM genes and thickening of the SCBMs in Alport mice. SCBM thickening results in hypoxic stress further elevating ECM and ET-1 gene expression, exacerbating strial pathology.

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Keywords

Alport syndrome; stria vascularis; basement membrane

Introduction

Alport syndrome is characterized by delayed onset and progressive glomerular disease associated with progressive, often high frequency hearing loss. It is the result of mutations in basement membrane type IV collagen genes. About 80 percent of the cases are X-linked and associated with mutations in the COL4A5 gene (Barker et al., 1990), and the remainder autosomal recessive, and associated with mutations in either COL4A3 or COL4A4 genes (Lemmink et al., 1994; Mochizuki et al., 1994). Mutations in any of these genes results in the absence of all three proteins in basement membranes where they are present, due to an obligatory association to form type IV collagen heterotrimers (LeBleu et al., 2010). The basement membranes where these collagens are normally found are now comprised of the ubiquitous collagen $\alpha 1(\text{IV})/\alpha 2(\text{IV})$ network in Alport patients.

In the glomerulus, and likely in the stria vascularis as well, this change in basement membrane composition results in a thinner structure with fewer interchain disulfide crosslinks (Gunwar et al., 1998). The consequences of this difference are two-fold. 1) The resulting capillary basement membrane is likely more elastic, which would be expected to impart higher than normal biophysical strain on glomerular cells, and 2) collagen $\alpha 1(\text{IV})/\alpha 2(\text{IV})$ networks (as opposed to collagen $\alpha 3(\text{IV})/\alpha 4(\text{IV})/\alpha 5(\text{IV})$ networks in normal mammals) are now in contact with glomerular podocytes, which may alter cell signaling. With regard to biomechanics, it has been shown in Alport mouse models that renal pathology is markedly accelerated in hypertensive compared to normotensive mice (Meehan et al., 2009) and measures of Young's modulus, which is a metric for the biophysical rigidity, is reduced in Alport glomeruli relative to wild type glomeruli (Wyss et al., 2011). With regard to cell signaling, it was recently shown using super resolution microscopy, that the collagen $\alpha 1(\text{IV})/\alpha 2(\text{IV})$ network in Alport mice is proximate to the podocyte pedicle surfaces, placing it in position to interact with podocyte cell surface receptors (Suleiman et al., 2013). One of these collagen receptors, the discoidin domain receptor 1 (DDR1), when mutated in mice, results in both GBM and SCBM dysmorphologies that are similar (but not identical) to those observed in Alport mice (Meyer zum Gottesberge et al., 2008; Gross et al., 2004). Another podocyte collagen receptor, integrin $\alpha 2\beta 1$, when mutated ameliorates the Alport glomerular phenotype, extending lifespan by 20% (Rubel et al., 2014).

Strial capillary basement membranes are thickened in Alport mice and demonstrate elevated levels of ECM proteins (Cosgrove et al., 1998; Gratton et al., 2005), similar to the Alport GBM (Cosgrove et al., 1996). Both the stria vascularis and the glomerulus show dysregulation of matrix metalloproteinases (Gratton et al., 2005; Rao et al., 2006; Cosgrove et al., 2008), and treatment of these mice with metalloproteinase inhibitors ameliorates glomerular disease progression (Rao et al., 2006; Zeisberg et al., 2006), suggesting that dysregulation of basement membrane homeostasis may play a significant role in the progression of the disease.

We previously showed that laminin 211 deposition in the GBM by mesangial filopodia activates focal adhesion kinase (FAK) on glomerular podocytes (Zalocchi et al., 2013; Delimont et al., 2014), and more recently that endothelin-1-mediated activation of the endothelin A receptors (ET_ARs) on glomerular mesangial cells activates the invasion of glomerular capillaries by mesangial filopodia (Dufek et al., 2016). Blocking either FAK or ET_ARs ameliorated the dysregulation of pro-pathologic genes, including matrix metalloproteinases (MMPs) in glomerular podocytes and significantly improved GBM architecture and function in these same mice. In the present study, we demonstrate that FAK is also activated in the strial capillaries and endothelin-1 is induced in the Alport stria. ET_ARs are present on strial marginal cells and basal cells. Laminin α 2, α 5, and type IV collagen are induced at both the mRNA and protein levels in Alport stria relative to wild type stria, and this matrix accumulation can be blocked by treating animals with the ET_AR antagonist, sitaxentan normalizing the SCBM thickness. We developed a strial marginal cell line to further explore this mechanism. Treating these cells with ET-1 induced the same ECM genes known to be elevated *in vivo*. Pre-treatment of these cells with sitaxentan prior to addition of ET-1 blocked induction of these genes. These same genes, including ET-1, are induced under conditions of hypoxia, in both the marginal cell culture system and *in vivo* in the stria vascularis. Our findings suggest that strial pathology and glomerular pathology in Alport mice share a common mechanism that hinges on ET_AR activation by ET-1.

Methods

Mice

129 Sv autosomal Alport mice were developed here (Cosgrove et al., 1996). The Alport mice used at the Gratton lab were derived from the Cosgrove colony. All mice were on a pure 129 Sv genetic background and maintained in house. The H-2Kb-tsA58 transgenic (immorto) mouse (Jackson Laboratories, Bar Harbor, ME) was used for strial explants to develop the strial marginal cell line. All procedures involving animals were conducted in accordance of an approved IACUC protocols at both sites: BoysTown National Research Hospital and Saint Louis University. Every effort was made to minimize usage as well as minimize any pain or distress.

Treatment of mice with sitaxentan

Sitaxentan (Sigma, St. Louis, MO) was solubilized in water and frozen in aliquots at 1 mg/ml. Animals were administered the drug at 10mg/kg daily by oral gavage from 2 weeks (pre-proteinuric) to 7 weeks (near end stage) of age.

Strial microdissection

A detailed description of the procedure for strial microdissection was described previously (Gratton et al., 2005).

Real time qRT-PCR analysis

Micro dissected striae were placed in Trizol™ (Ambion) and RNA isolated utilizing Direct-zol™ RNA Mini Prep Kit (Cat. # R2050 ZYMO Research, Irvine, CA). cDNA (from cells and stria) was generated using Superscript®III First-Strand (Cat. #18-080-05

Invitrogen, Waltham, MA). qPCR was run with the following TaqMan[®] Gene Expression Assays (Cat.# 4331182 Applied Biosystems, Waltham, MA) : Col4 α 1 Mm0120125_m1, ET-1 Mm00438656_m1, GLUT 1 Mm0441473_m1, HIF1 α Mm00468869_m1, Lama1 Mm01226102_m1, Lama2 Mm01193171_m1, Lama5 Mm01222046_g1, NFKBia Mm0477798_m and VEGF1 Mm01281449_m1.

Noise exposure

A subgroup of randomly selected Alport and wildtype mice were exposed to a moderate noise with a 106 dB SPL, octave band of noise (OBN) centered at 10 kHz for 10 hours. Immediately prior to exposure, the mice were hydrated with 2ml sterile lactated Ringers s.c. and are then placed into individual wire-mesh cages containing food. Following the noise exposure, the mouse was removed for physiological assessment (ABR) or was euthanized for ultrastructural or biochemical analysis of the cochlear lateral wall.

ABR Testing

Animals were sedated (Avertin [2,2,2-Tribromoethanol, Sigma Aldrich, St. Louis, MO] 0.4 mg/gm i.p.) with supplemental injections (0.5 original dose) as needed. Anesthetized animals were placed in a head holder with the right ear directed superiorward in an acoustically shielded sound chamber. Body temperature was maintained at 37°C using a thermoelectrical feedback system (FHC, Bowdoin, ME). Heart function was monitored auditorily. Tone pips at half-octave intervals (8-32kHz) were synthesized, shaped (1 ms cosine ramps, 2 ms plateau), and delivered via the Tucker-Davis ABR workstation. The stimuli were delivered via a customized high frequency transducer at a rate of 12/sec. Sound pressure levels (dB SPL) of the tone pips were calibrated using a 6.25 mm condenser microphone (ACO-Pacific, Belmont, CA) positioned at the approximate location of the animal's head. The ABR was recorded using needle electrodes positioned subdermally at the vertex (active, noninverting), at the bulla (reference, inverting) and over the musculature of the rump (ground). Differentially recorded voltages were amplified, filtered (30 Hz – 3 kHz), digitized, and averaged using the Tucker-Davis ABR Workstation software. Each ABR waveform was averaged over a 16 ms time window and resolved after 512 – 1024 samples. Artifact rejection was used to enhance reliability and validity of the ABR. The intensity of the tonal stimuli was decreased in 10 dB steps and varied in 5 dB steps for stimuli near threshold. Threshold was defined as half the interval between lowest stimulus level producing a replicable response waveform and the stimulus level below which no reproducible response is elicited.

A subgroup of randomly selected Alport and wildtype mice underwent ABR testing prior to, immediately after (Day 0) and five days (Day 5) after exposure to the moderate noise

Immunofluorescence analysis

Cochleae were perfused with 4% paraformaldehyde and then decalcified overnight in 150mM EDTA on a rotator at 4°C. The samples were transferred to 15% sucrose for 1 hour and then into 30% sucrose for 2 hours. Samples were mounted in OCT and frozen at –80°C. Cochleae were sectioned at 8- μ m and dipped in cold acetone. After air drying 2 hours, slides were rehydrated with 1X PBS. Cochleae sections used for collagen IV immunostaining were

first denatured with acid urea (6M urea, 0.1M glycine, pH 3.5) for 1 hour at 4°C and then rinsed with 1X PBS. Slides were stained with one of the following antibodies: rat anti-mouse Laminin $\alpha 2$ antibody (L0663, Sigma-Aldrich, St. Louis, MO, USA) at 1:500, rabbit anti-mouse Laminin $\alpha 5$ antibody (a gift from Jeff Miner, Washington University) at 1:1500, rabbit anti-mouse Phospho-FAK³⁹⁷ antibody (44-625G, Thermo Fisher Scientific, Waltham, MA, USA) at 1:25, rabbit anti-mouse Collagen $\alpha 1IV$ antibody (T40261R, Biodesign, Saco, ME, USA) at 1:500 or sheep anti-mouse Endothelin R Type A antibody (NB600-836, Novus Biologicals, Littleton, CO, USA) at 1:200. Sections were incubated overnight at 4°C in a humidified chamber with their primary antibodies diluted in 0.1% PBST (Triton X-100) + 5% FBS. Slides were rinsed with 1X PBS and incubated with the appropriate Alexa Fluor secondary antibodies at 1:500 for 1 hour at room temperature. The slides were rinsed again with 1X PBS and mounted with Vectashield Mounting Medium with Dapi (H-1200, Vector Laboratories, Burlingame, CA, USA).

Confocal Microscopy

Confocal images captured using a Leica TCS SP8 MP confocal imaging system, using a 63x NA: 1.4 oil or 10x NA: 0.3 objective. Final figures were assembled using Adobe Photoshop and Illustrator software (Adobe Systems, CA).

Transmission Electron Microscopy

Preparation of the cochlea for transmission electron microscopy was conducted as described previously (Gratton et al., 2005). Thin sections (70 nm) of the scala media cut in the mid-modiolar plane were mounted on copper grids, counterstained with uranyl acetate and lead citrate and examined at 40k magnification in a Hitachi H-7500 transmission electron microscope. Digitized images were obtained and archived with an ORCA camera with IC-PCI framegrabber and AMT 12-HR software. Measurements of basement membrane width were made at approximately 500nm intervals around the stria capillary profile.

Derivation of Strial marginal Cells

The micro dissected stria vascularis from an H-2Kb-tsA58 transgenic (immorto) mouse cochlea was cut into several pieces and placed onto BD Cell Tak™ (BD Biosciences, Bedford, MA.) coated- tissue culture dishes. Outgrowths were propagated at 33°C in DMEM/F-12 Media (Gibco, Grand Island, NY.) supplemented with 15% heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA.), 50 units/ml mouse recombinant γ -interferon (Calbiochem, La Jolla, CA.) and penicillin, streptomycin, glutamine (Gibco). Outgrowths were dissociated with 0.05% trypsin, 0.02% EDTA (Gibco) and cloned by limiting dilution. Fifty clones were subsequently frozen back, twenty of which were expanded for further characterization by RT-PCR and immunofluorescence. To expand, sub confluent (80%) clonal cultures were dissociated and replated 1:5 in 10% FBS, 10 U/ml γ -interferon containing medium on dishes coated with rat tail Collagen Type 1 (Corning, Bedford, MA.) at 33°C. To induce differentiation, dissociated cells were plated in 5% FBS-containing medium without γ - interferon, placed at 37°C for at least seven days.

Hypoxia Induction

Sub-confluent Strial Marginal cells plated on Collagen Type 1 from rat tail (Corning, Bedford, MA.) were differentiated (9 days at 37°, 5 % FBS-containing media, less α -interferon) placed on 0.5% FBS-containing media overnight and the next day placed in a hypoxia chamber (Cat.#27310; Billups-Rothenburg, Dell Mar, CA). Utilizing two independent flow meters (Billups-Rothenburg) attached separately to compressed nitrogen and carbon-dioxide gas, the chamber was flushed for 10 minutes with 95% \uparrow N₂/ 5% \uparrow CO₂ at a flow rate of ~20 liters/min. Immediately after purging the chamber was sealed and placed in a conventional CO₂ incubator. The system was re-flushed after 1 hour, as recommend by the manufacturer. RNA was collected, at indicated time points, by removing cultures from chamber and immediately applying TRIzol[®] Reagent (Ambion, TX) to cells. Normoxia control culture(s) were placed in an identical chamber left “open” to CO₂ incubator conditions.

Sitaxentan/ET-1 Treatment of strial marginal cells

Differentiated Strial Marginal cells were exposed to 10 μ M Sitaxentan (Sigma, St Louis, MO.) for one hour and then treated with 1 μ g/mL ET-1 (Cat.# 55553 Syd Labs, Boston, MA.). Non-treated control and ET-1 alone treatment were also performed. RNA was collected, at indicated time points, as before, and analyzed by real time RT-PCR for ECM transcripts.

Statistical analysis

The measures of SCBM thickness were subjected to 1-way ANOVA analysis with the factor of genotype/treatment (, Kruskal-Wallis, *SigmaStat, Jandel, San Jose, CA*) followed by post-hoc multiple comparisons (Dunn's Method). For ABR analysis, hearing loss incurred at each timepoint for the Alport mice and their age-matched WT controls was subjected to ANOVA with factors of test frequency, and strain/test day with post-hoc comparisons made using the Holm-Sidak test (SigmaStat, Jandel, San Jose, CA). Significance was set at a p value 0.05. For all other analyses, one or two way (as appropriate) Student's t-test was used. Significance was set at the probability level of 0.05

Results

Strial capillary basement membrane thickening in the Alport mouse model was reported previously (Cosgrove et al., 1998; Gratton et al., 2005). In the study by Gratton et al. (2005), colloidal gold TEM was used to show that collagen α 1(IV)/ α 2(IV), laminin α 1, and laminin α 5 are present at higher levels in the Alport stria compared to wild type controls. Laminin 211, which was shown to activate FAK, resulting in a pro-inflammatory signal cascade in glomerular podocytes was never examined in this study (Delimont et al., 2014). To address this, mid-modiolar cross sections of wild type and Alport cochleae were dual immunostained using antibodies specific for either laminin α 2 chain or activated FAK (pFAK³⁹⁷). The lateral walls are shown in **Figure 1A**. It is clear that laminin α 2 is up-regulated in the Alport stria vascularis in the regions surrounding the strial capillaries, where it co-localizes with pFAK³⁹⁷. This is similar to what we have previously documented in the renal glomerulus (Delimont et al., 2014). **Figure 1B** shows that the I kappa B-alpha (NFkappaBIA) mRNA

expression in isolated stria from Alport mice is elevated relative to wild type mice, consistent with pro-inflammatory NFkappaB activation in the Alport stria (Bottero et al., 2003).

Alport mice show only mild progressive hearing loss (Cosgrove et al., 1998). We surmised that if thickened strial capillary basement membranes altered vascular permeability, the strial cells might be predisposed to metabolic stresses. To test this, we subjected wild type and Alport mice to noise exposure (8-17 kHz, 106 dB SPL) for 10 hrs. Hearing loss was assessed immediately post noise, and again at 5 days post noise by auditory brainstem response (ABR). **Figure 2A** shows that immediately post noise the Alport mice show significantly elevated thresholds (2-way ANOVA: $F_{(9,92)} = 4748.774$, $p < 0.001$) at the middle frequency ranges (16 and 24 kHz), when compared to wild type mice ($p < 0.05$, Holm-Sidak). **Figure 2B** shows that by 5 days post-noise exposure, wild type mice have returned to normal thresholds, while Alport mice again show significantly elevated thresholds at 16 and 24 kHz ($p < 0.05$, Holm-Sidak), likely reflecting permanent noise-induced damage.

More recently we have shown that endothelin-1 (ET-1) activates the accumulation of abnormal GBM laminins (including laminin 211) and initiates epithelial cell dysfunction in the renal glomerulus via activation of the endothelin A receptor subclass (Dufek et al., 2016). Blocking ET_ARs using sitaxentan resulted in the inhibition of ECM accumulation in the GBM and amelioration of glomerular disease initiation and progression. We analyzed expression of ET-1 and ECM genes encoding proteins known to accumulate in the SCBM of Alport mice using RNA isolated from microdissected stria vascularis from 7 week old wild type and Alport mice plus or minus noise exposure as described above. The results in **Figure 3A** show that both ET-1 mRNA and mRNA encoding ECM proteins known by immunogold analysis to accumulate in Alport stria (Gratton et al., 2005), are significantly higher in Alport stria vascularis compared to stria from age/strain matched wild type mice. Furthermore, exposure of wild type mice to noise significantly elevates these same transcripts. Interestingly, while expression of these transcripts in the stria from Alport mice exposed to noise is still elevated compared to wild type mice under quiet conditions, Alport noise is actually lower than Alport quiet, again likely reflecting noise-induced damage to the strial cells.

Thickened SCBMs would be expected to impart metabolic stresses leading to a hypoxic environment in the stria vascularis. Metabolic stresses caused by intense noise exposure have been documented in the stria vascularis, and lead to upregulation of hypoxia inducible factor 1 α (Hif1- α) and vascular endothelial growth factor (VEGF) (Shi, 2009). We compared stria vascularis from non-noise exposed and noise exposed wild type mice to stria vascularis from non-noise and noise-exposed Alport mice for expression of mRNA encoding Hif1- α and VEGF. Glucose transporter-1, expressed by strial capillary endothelial cells (Ando et al., 2008), was also measured, as it would be expected to be up-regulated under conditions of metabolic stress (Barnes et al., 2002). The results in **Figure 3B** show that all three transcripts are significantly elevated in stria vascularis from both non-noise exposed Alport mice and noise exposed wild type mice compared to non-noise exposed wild type mice, suggesting that the SCBM thickening in the Alport stria vascularis results in a hypoxic microenvironment which results in metabolic stress. Like the ECM and ET-1 transcripts,

expression hypoxia-related genes were lower in stria from noise-exposed Alport mice compared Alport mice from quiet conditions. This may reflect the already stressed condition of the stria in Alport mice, and along with the data for these same mice in **Figure 3A**, may reflect damage to the strial cells. These observations in Alport quiet versus Alport noise stria are consistent with the permanent shift in ABR thresholds observed in **Figure 2B**.

In the renal glomerulus, ET-1 mediated ECM and pro-inflammatory cytokine induction is regulated by ET_AR signaling. Blocking ET_ARs using the small molecule inhibitor, Sitaxentan, ameliorates glomerular and renal disease in Alport mice (Dufek et al., 2016). We examined the cellular distribution of ET_ARs in the stria vascularis in adult mice by immunohistochemistry. The antibodies would only work in unfixed tissue, so freshly microdissected stria vascularis was used. **Figure 4** demonstrates that only the strial marginal cells and basal cells express ET_ARs. To address the role of ET-1 mediated ET_AR activation in the regulation of ECM genes we developed a strial marginal cell line from the immortomouse. Strial explants from adult mice were adhered to tissue culture plates using Cell-Tak (Corning, Inc.) and outgrowths cloned twice. Individual clones were characterized by real time PCR using primers to detect the strial cell-specific markers. One clone was selected that was positive for all the strial marginal cell-specific markers and expressing very low to undetectable levels for intermediate/basal cells markers shown in **Table 1**. This clone was expanded, differentiated, and stimulated with ET-1 in the presence or absence of the ET_AR receptor antagonist, sitaxentan. RNA from these cells was then analyzed by real time RT-PCR for the ECM proteins shown in **Figure 5A**. The results show that the transcripts encoding the ECM proteins known to accumulate in the Alport stria vascularis are expressed in strial marginal cell cultures and are up-regulated by stimulating the cells with ET-1. Co-incubation of ET-1 with sitaxentan significantly reduced expression of these same ECM transcripts, supporting the notion that ET-1 regulates ECM expression in strial marginal cells via ET_AR signaling.

We exposed these same cells to hypoxic conditions to determine whether hypoxia (metabolic stress) itself might contribute to the induction of genes encoding ECM proteins, as is suggested from the *in vivo* noise exposure studies (**Figure 3B**). The results in **Figure 5B** show that hypoxia related genes (Hif1- α and VEGF) were induced in cells cultured under hypoxic conditions. Messenger RNAs for both ET-1 and ECM proteins were also significantly induced, suggesting that once established, the metabolic stresses in Alport stria might themselves contribute to the SCBM thickening and stria pathology.

Lastly, we aimed to determine whether ET_AR blockade *in vivo* would prevent the accumulation of ECM proteins in the SCBM as well as prevent SCBM thickening. Wild type and Alport mice were treated with sitaxentan (10 mg/kg) or vehicle from 2 to 7 weeks of age. One ear was processed for transmission electron microscopy and the other processed for immunofluorescence analysis. The results in **Figure 6** show that collagen α 1(IV), laminin α 2, and laminin α 5 are all significantly elevated in the Alport stria relative to the wild type stria, and treatment with sitaxentan prevents the accumulation of these matrix proteins in the SCBMs. It is notable in these immunostains that immunostaining intensity in the capillaries of the spiral ligament is not affected by sitaxentan treatment. TEM analysis shows that untreated Alport SCBMs are significantly thicker ($H =$, 1227.979, 1 df, $p = <0.001$ than that

for either treated or untreated wild type mice. Sitaxentan treatment prevents SCBM thickening (**Figure 7A**). Figure 7B shows an example of the differences in SCBM thickness for the wildtype and Alport mice; the black bars delineate the BM width and illustrate how the SCBM morphometric measures were performed. Collectively the data in **Figures 6 and 7** suggest SCBM thickening is caused by ET_AR-mediated ECM gene dysregulation in strial marginal cells, which underlies SCBM thickening and strial dysfunction.

Discussion

This study demonstrates that progressive SCBM thickening, which results in metabolic and oxidative stress in the stria vascularis of Alport mice, evolves through a mechanism that is remarkably similar to the mechanism of glomerular basement membrane disease (Dufek et al., 2016); Endothelin-1 mediated activation of ET_ARs on strial marginal cells resulting in elevated expression of ECM proteins that accumulate in the SCBMs. We show using both *in vivo* and *in vitro* systems, that ET_AR blockade results in reduced ECM protein expression, normalization of SCBM thickness and reduced expression of mRNAs encoding ECM proteins that accumulate in the SCBMs. The link between ET-1-mediated ET_AR activation and ECM protein accumulation in fibrotic mechanisms is well established (Shi-Wen et al., 2001; Xu et al., 2004), however attempts to control or reverse the fibrotic process by way of ET_AR blockade have produced disappointing results (Rodriguez-Pascual et al., 2014). In the Alport mouse model ET_AR blockade produced impressive results in preventing SCBM thickening and ECM accumulation in the SCBMs.

Strial pathology associated with Alport syndrome has been understudied relative to Alport glomerular pathology and the literature on inner ear pathology associated with Alport syndrome is not without controversy. The only ultrastructural defect observed in the Alport mouse cochlea was a thickening and lamination of the strial capillary basement membranes associated with endothelial cell swelling (Cosgrove et al., 1998). This defect is very similar to one described in humans with Alport syndrome many years ago (Weidauer et al., 1976). More recently in a study conducted on archived celloidin-embedded human temporal bones from Alport patients, the authors concluded that a shared defect of splitting of the basilar membrane and cellular infilling of the tunnel of Nuel might predict a defect in cochlear mechanics (Merchant et al., 2004). One important difference between the two studies is that Weidauer and Arnold fixed the tissue within one hour of death, immediately removed the membranous tissue and prepared it for transmission electron microscopy.

It is important to note that Merchant *et al.* (2004) claimed not to observe SCBM thickening in the human samples. What the authors failed to recognize is that the SCBM has two layers; an inner layer adjacent to the endothelium and an outer layer that lies on the outer surface of the pericytes (Neng et al., 2013). The outer layer, which forms contacts with strial marginal cell basolateral infoldings, is thickened in the Alport mice (Cosgrove et al., 1998). This layer is also thickened in the samples from Alport patients as is clearly evident in Merchant *et al.*, and in Weidauer and Arnolds study.

Lastly, if defective cochlear mechanics were responsible for hearing loss in human Alport patients, as hypothesized by Merchant *et al.* (2004), one would expect that ALL Alport

patients with hearing loss would have impaired otoacoustic emissions (OAEs). Moon *et al.* (2009) found that OAEs had no predictive value in young Alport patients with documented hearing loss. As patients get older OAEs do change, likely due to hair cell loss, as documented in Merchant's study.

Our findings may have broader relevance than Alport syndrome. Strial capillary basement membrane thickening has been associated with diabetic hearing loss in humans (Akinpelu *et al.*, 2014) and implicated in presbycusis, where strial atrophy is often observed (Nelson *et al.*, 2006). In aging gerbil models thickening of the strial capillary basement membranes is observed and associated with the accumulation of laminin (Gratton *et al.*, 1996; Thomopoulos *et al.*, 1997; Sakaguchi *et al.*, 1997). It would be of interest to explore whether the ET-1/ET_AR axis for ECM dysregulation plays a role in SCBM thickening in models for these forms of hearing loss.

ET-1 mediated ET_AR activation is the earliest event we have documented to be associated with glomerular pathology in Alport mice. The fact that this pathway appears activated in the stria vascularis in Alport mice is remarkable and suggests the possibility for the identification of single-target therapeutic approaches that may attenuate/arrest both the glomerular and strial pathologies in their pre-initiated state. This approach has the potential for preserving both hearing and kidney function in patients afflicted with the disease. Studies are under way aimed at determining whether endothelin blockade in Alport mice results in normalization of hearing, susceptibility to noise-induced hearing loss, endocochlear potentials, and measures of metabolic stress. These studies will define whether ET_AR antagonism is a potential therapeutic strategy to prevent hearing loss associated with Alport syndrome.

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Highlights

- Strial capillary basement membrane thickening underlies cochlear pathology in Alport syndrome.
- Endothelin-1 up-regulates genes encoding ECM molecules in strial marginal cells.
- blocking the endothelin A receptor in vitro or in vivo prevents elevates expression of ECM components in the strial capillary basement membranes and ameliorates SCBM thickening in the mouse model.

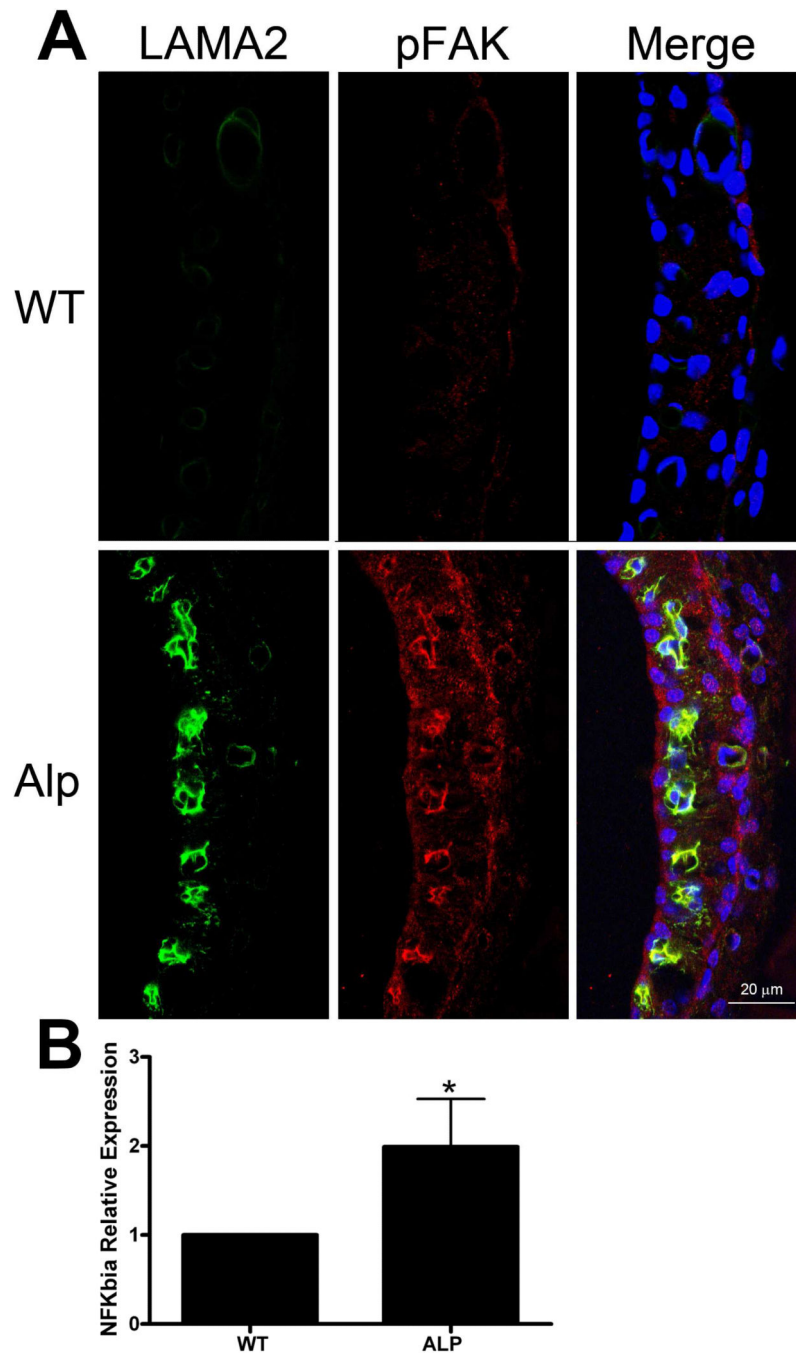


Figure 1. Laminin 211 accumulates in the SCBM of Alport mice activating FAK and pro-inflammatory NF-kappaB. **Panel A:** Wild type and Alport stria vascularis from 7 week old mice were immunostained using antibodies specific for laminin α 2 chain and antibodies that detect the activated form of FAK, pFAK³⁹⁷. **Panel B:** RNA from wild type and Alport stria vascularis was analyzed for the NF-kappaB1a transcript by real time RT-PCR. Asterisk denotes statistical significance ($P < 0.05$).

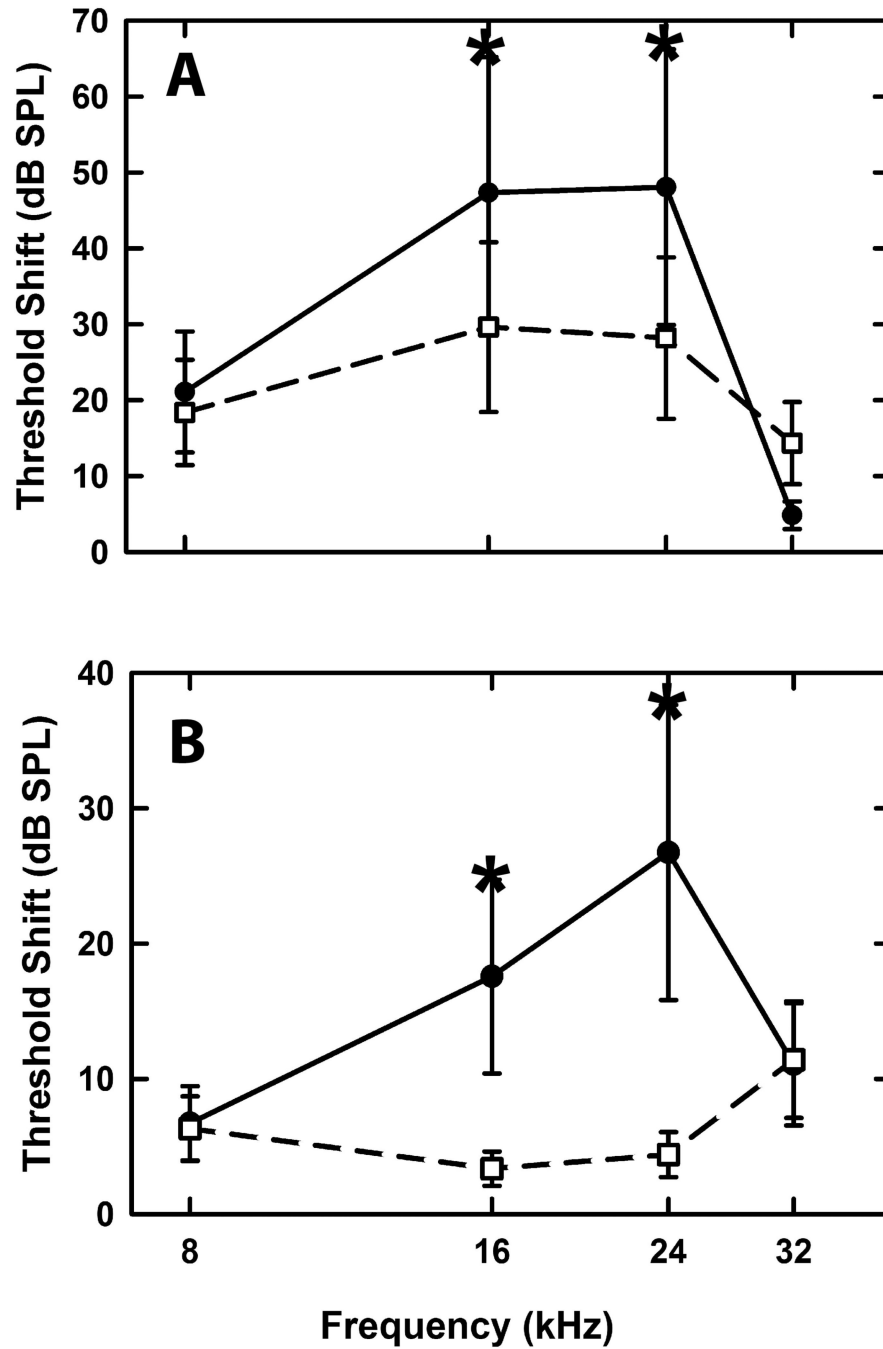


Figure 2. Noise exposure results in significantly elevated thresholds for Alport mice relative to wild type mice. Mice were exposed to noise (8-17 kHz, 106 dB SPL) for 10 hrs. Hearing was assessed using the ABR immediately post-noise exposure (**panel A**) and again at 5 days post-noise exposure (**panel B**). Asterisks denote frequencies that contributed to the statistically significant differences noted overall between the wild type and Alport mice ($P < 0.05$).

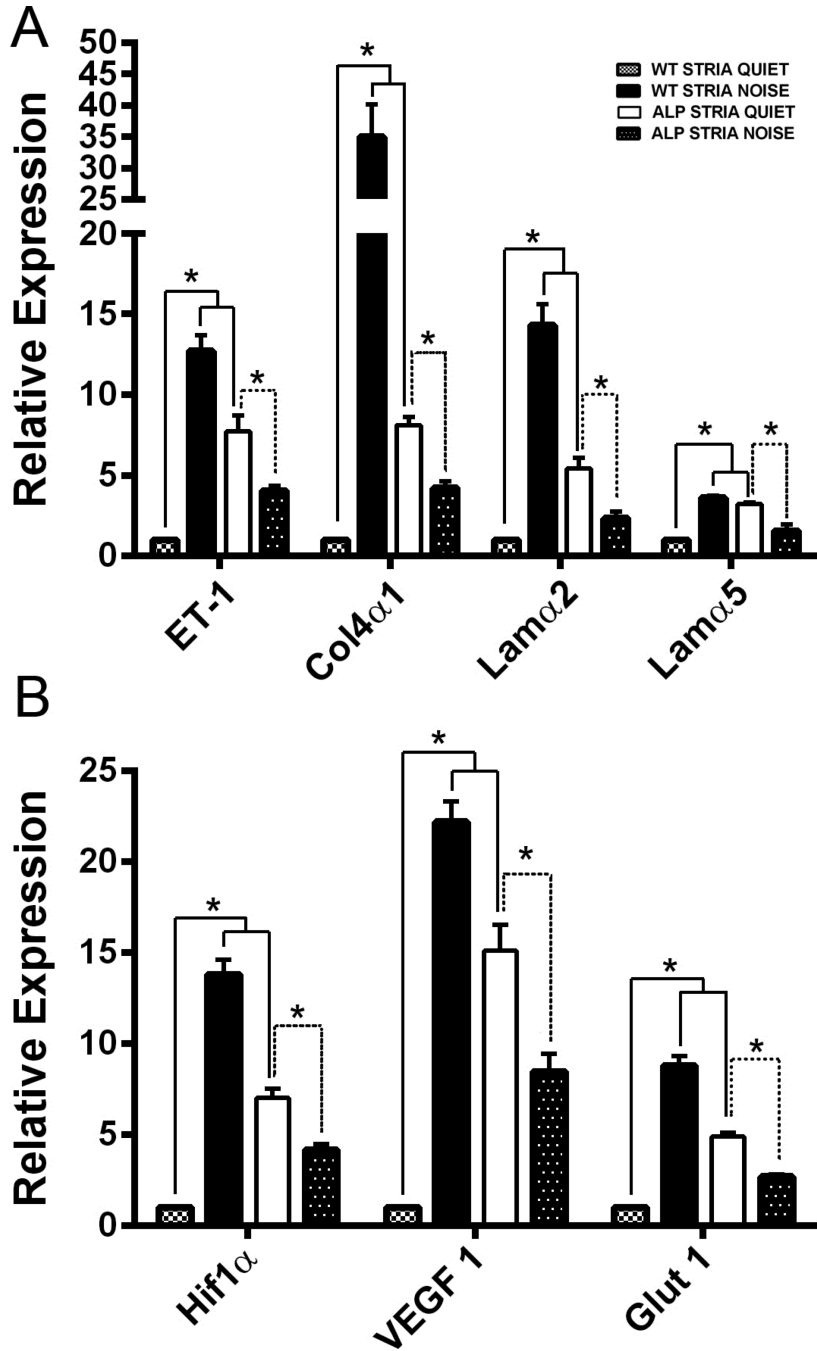


Figure 3. The stria vascularis in Alport mice is under metabolic/oxidative stress and overexpresses transcripts encoding ECM molecules. Stria vascularis was isolated from 7 week Alport mice, strain and age-matched wild type mice, and wild type mice exposed to noise (see methods). RNA was analyzed by real time RT-PCR for transcripts encoding endothelin-1, the ECM molecules known to accumulate in the SCBM (laminin α 2, laminin α 5, and collagen α 1(IV), **Panel A**), or for hypoxia related factors Hif-1 α and VEGF or glucose transporter-1

(Glut1) (**Panel B**), which is up-regulated under conditions of metabolic stress. Asterisks denotes statistically significant differences ($P < 0.01$).

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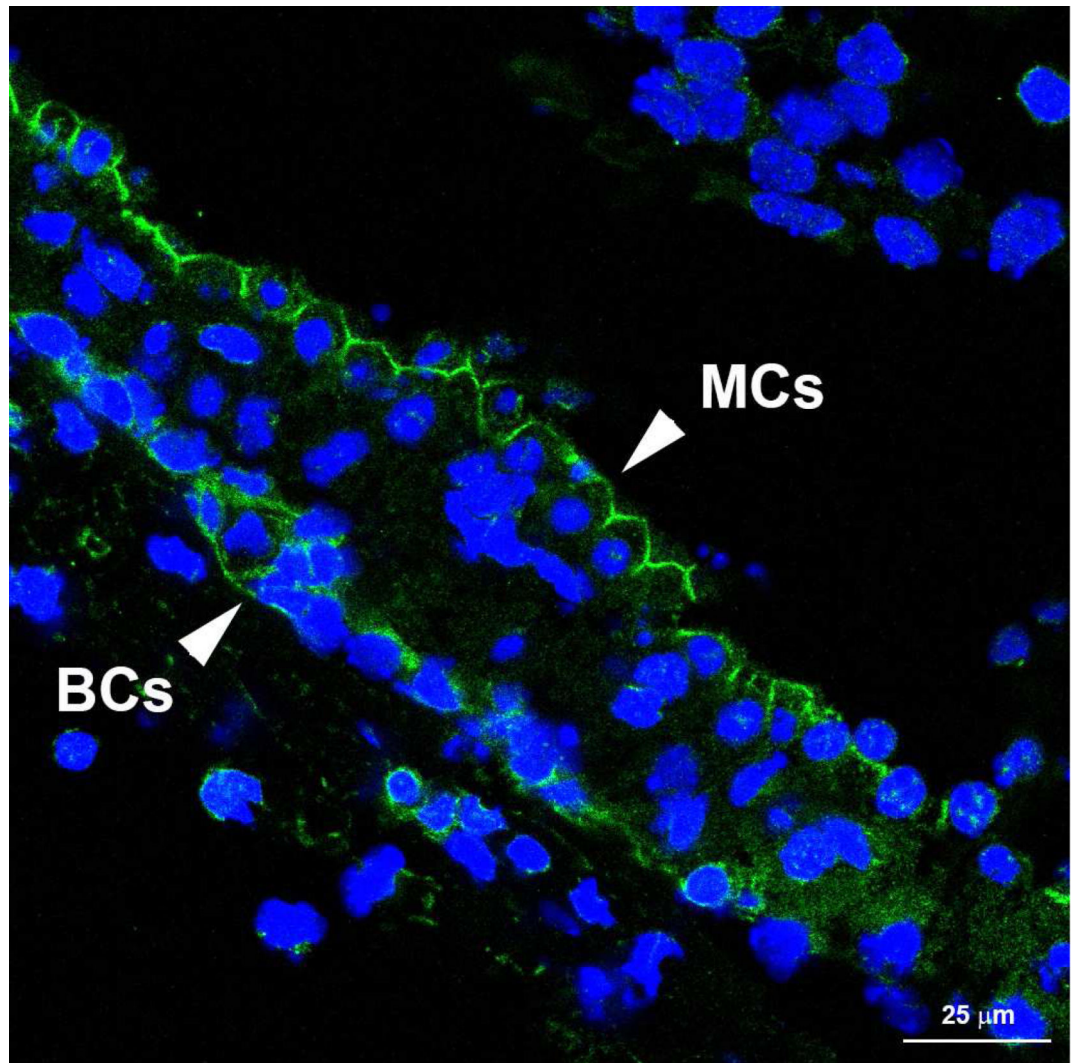


Figure 4. Endothelin A receptors are expressed by strial basal cells and strial marginal cells. The lateral wall with the intact stria vascularis was dissected from unfixed cochlea of a 4 week old wild type mouse and cryosections immunostained using antibodies specific for ET_ARs. Both marginal cells (MCs) and basal cells (BCs) were immunopositive.

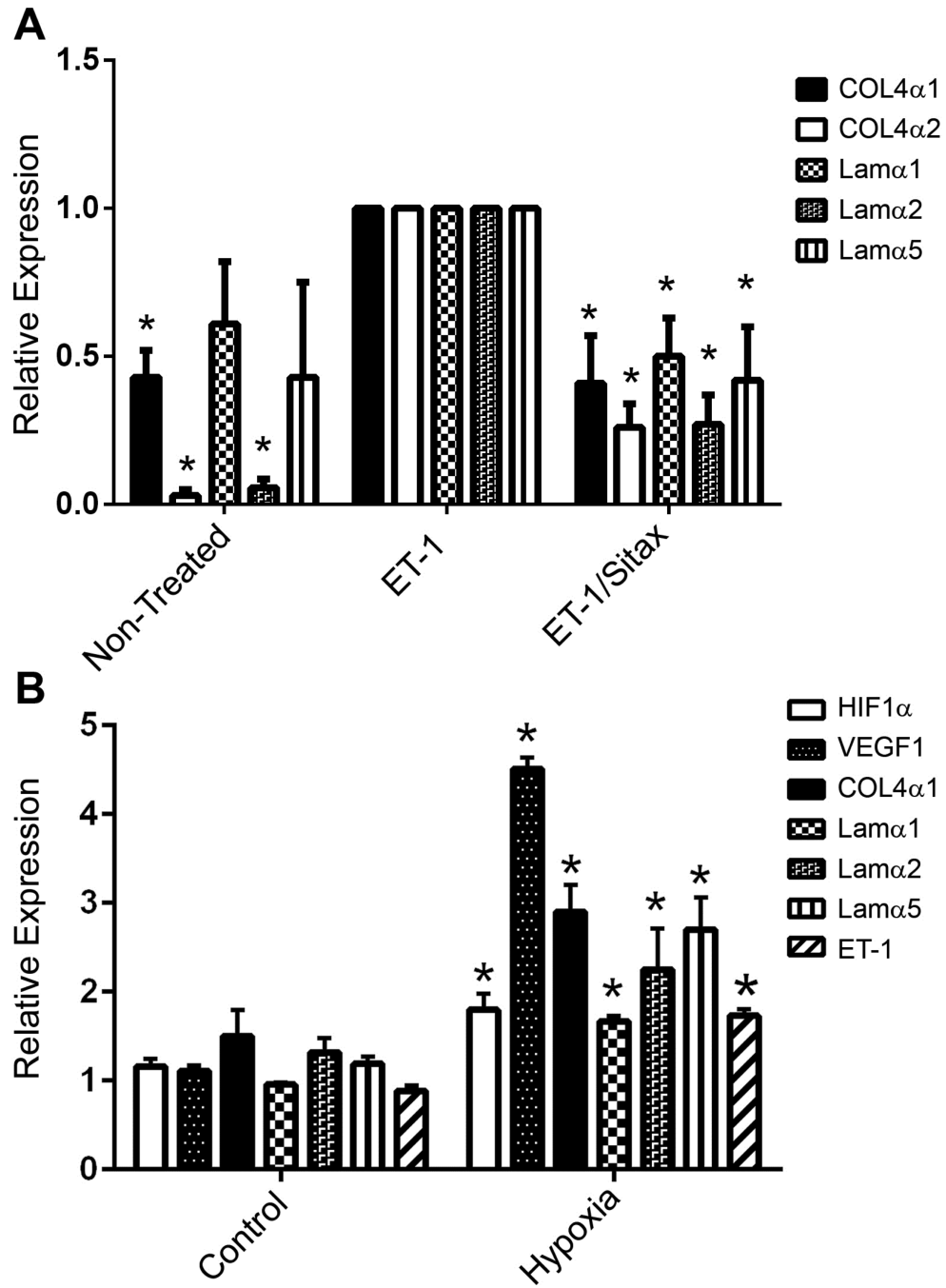


Figure 5. Stria marginal cells respond to both ET-1 and hypoxia by up regulating expression of ECM genes, and the response can be blunted by ET_AR blockade. **Panel A.** Differentiated strial marginal cell cultures were stimulated with ET-1 in the presence or absence of sitaxentan. RNA was analyzed for the indicated ECM transcripts. **Panel B.** Differentiated strial marginal cells were cultured under normoxic or hypoxic conditions (for 24 hours) and RNA analyzed for the indicated transcripts. Asterisks denote statistically significant differences (P<0.05).

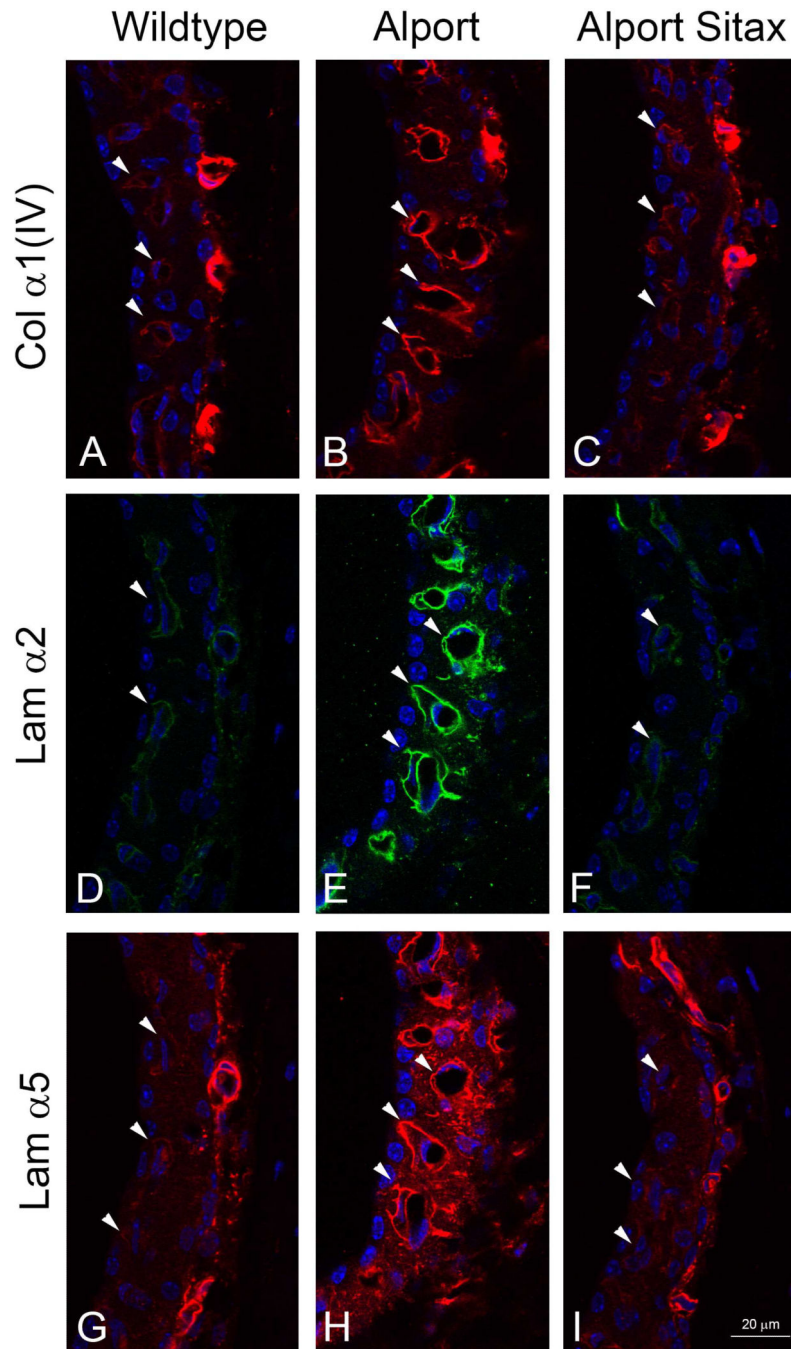


Figure 6. Endothelin A receptor blockade prevents accumulation of ECM in the Alport strial capillary basement membranes. Alport mice were treated with sitaxentan or vehicle from 2 weeks to 7 weeks of age. Mid-modiolar cryosections were immunostained with antibodies against type IV collagen $\alpha 1$ chain (col $\alpha 1$ (IV)), laminin $\alpha 2$ chain (Lam $\alpha 2$), or laminin $\alpha 5$ chain (Lam $\alpha 5$). All sections were stained at the same time and microscope settings standardized to the vehicle-treated Alport cochleae. Arrow heads denote strial capillary basement membranes.

Note that vessels in the spiral ligament do not show changes in staining intensity upon sitaxentan treatment, and thus serve as a control.

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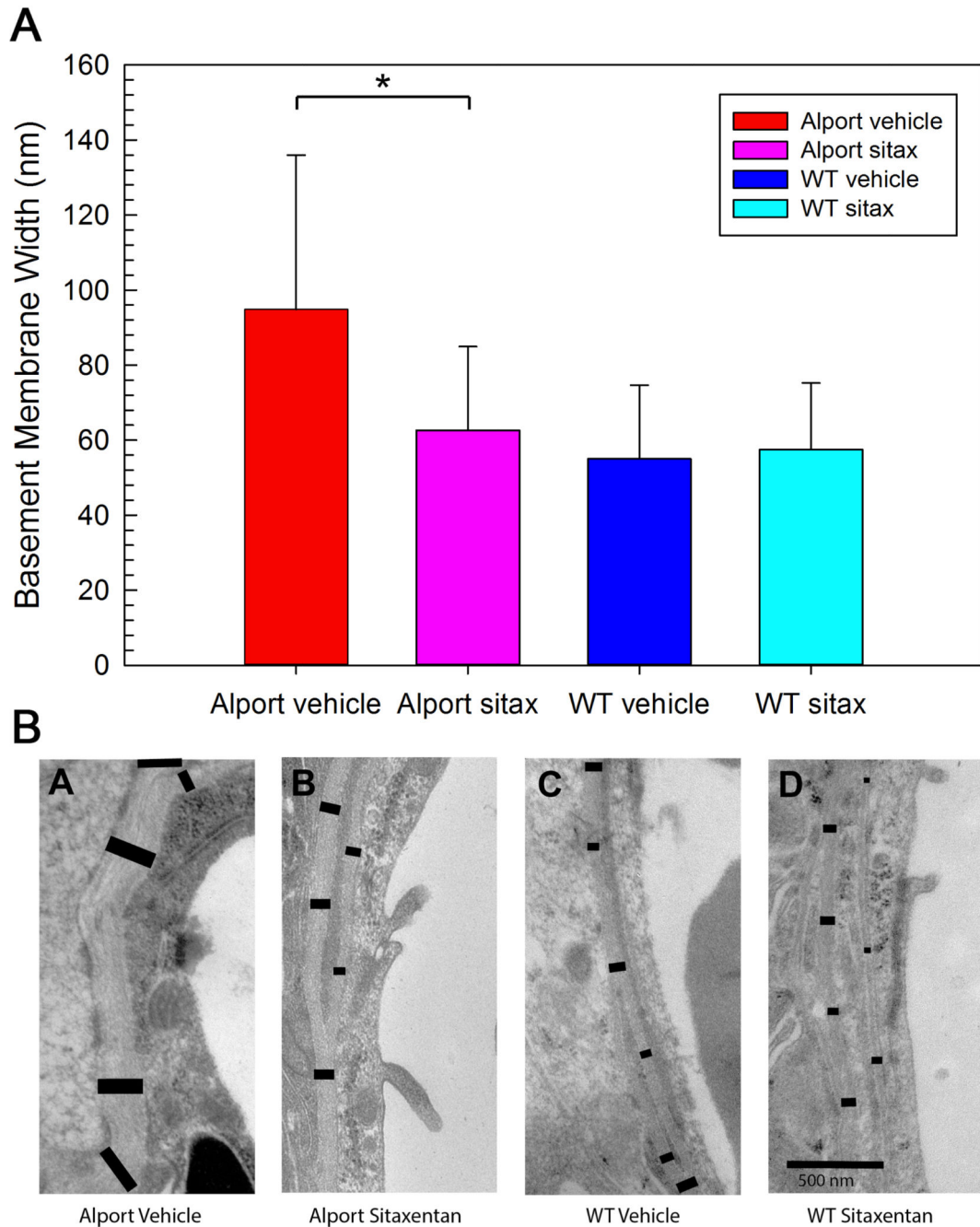


Figure 7. Endothelin blockade ameliorates strial capillary basement membrane thickening in Alport mice. **Panel A.** Morphometric analysis of strial capillary basement membrane thickness was done using transmission electron micrographs for multiple capillaries from at least 4 independent animals per group. **Panel B.** An example of the methodology used for morphometric analysis of SCBM thickness. Asterisk denotes a statistically significant difference ($P < 0.05$).

Table 1

RT PCR Markers for Marginal Cell Clone Characterization.

Transcript	Strial Cell Expression	Reference
- Connexin 26	Basal/Intermediate	Liu YP, et al. 2008
+ Del1	Marginal	Giroto G, et al. 2014
- Glut-1	Basal/Endothelial	Barnes K, et al. 2002
+ KCNQ 1	Marginal	Hur DG, et al. 2007
+ KCNE 1	Marginal	Hur DG, et al. 2007
- KCNJ10 (Kir4.1)	Intermediate	Jin Z, et al. 2006 & Chen J, et al. 2014
+ KvLQT1 (IsK)	Marginal	Teixeira M, et al. 2006 & Tu TY, et al. 1999
+ Na, K- ATPase	Marginal	Crouch JJ, et al. 1997
- PANx2	Basal	Wang XH, et al. 2009
+ SIRT1	Intermediate/ Marginal	Takumida M, et al. 2014
+ KCNK1 (TWIK-1)	Marginal	Nicolas MT, et al. 2003
+ ZO-1	Marginal	Suzuki T, et al. 2001

- = low expression; + = high expression

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