Published in final edited form as:

Exp Eye Res. 2013 November ; 116: 419–423. doi:10.1016/j.exer.2013.10.017.

Smad3 is necessary for transforming growth factor-beta2 induced ocular hypertension in mice

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Abstract

TGFβ2 induces extracellular matrix (ECM) remodeling and alters the cytoskeleton by both the canonical Smad and non-canonical signaling pathways. TGFβ2 regulates the expression of ECM proteins in trabecular meshwork (TM) cells, increases intraocular pressure (IOP) in an *ex vivo* perfusion organ culture model, and induces ocular hypertension in rodent eyes. A necessary step in the canonical Smad signaling pathway is phosphorylation of receptor protein Smad3 by the TGF-β receptor complex. The purpose of this study was to determine whether TGFβ2 signals *in vivo* through the canonical Smad signaling pathway in the TM using Smad3 knockout (KO) mice. Ad5.hTGFβ2^{226/228} (2.5×10^7 pfu) was injected intravitreally into one eye of homozygous (WT), heterozygous (HET), and homozygous (KO) 129-Smad3^{tm1Par/J} mice (n=9–10 mice/group), with the uninjected contralateral eye serving as the control. IOP measurements were taken using a rebound tonometer. To test the effect of TGFβ2 signaling on the ECM, fibronectin expression was determined by immunohistochemistry and qPCR analysis. Transduction of the TM with viral vector Ad5.hTGFβ2^{226/228} caused a statistically significant difference in IOP exposure between Smad3 genotypes: WT, 187.7 ± 23.9 mmHg*day (n=9); HET, 95.6 ± 24.5 mmHg*day (n=9); KO, 52.8 ± 25.2 mmHg*day (n=10); (p<0.05 WT versus HET, p<0.01 WT versus KO). Immunohistochemistry and qPCR analysis showed that Ad5.hTGFβ2^{226/228} increased fibronectin expression in the TM of WT mice (2.23 ± 0.24 fold) compared to Smad3 KO mice (0.99 ± 0.19 fold), p<0.05. These results demonstrate Smad3 is a necessary signaling protein for TGFβ2-induced ocular hypertension and fibronectin deposition in the TM.

Keywords

glaucoma; mouse model; ocular hypertension; TGFβ2 signaling; trabecular meshwork

The glaucomas are a heterogeneous group of optic neuropathies sharing similar clinical features including cupping of the optic disc, thinning and loss of the retinal nerve fiber layer, and characteristic visual field defects (Quigley, 1993). Risk factors for developing glaucoma include elevated intraocular pressure (IOP), age, family history, central corneal thickness, and steroid responsiveness. IOP is the most significant causative risk factor for both the development and progression of glaucoma.

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IOP is regulated by aqueous humor production and drainage from the eye. The trabecular meshwork (TM) is well known to be a critical tissue in aqueous humor drainage. The extracellular matrix (ECM) composition of the TM plays a major role in the regulation of IOP. Increased deposition of ECM proteins in the TM, increased AH outflow resistance, and increased IOP are associated with primary open angle glaucoma (Lutjen-Drecoll, 1999; Rohen and Witmer, 1972). These data demonstrate that the ECM architecture of the TM is important in regulating aqueous humor outflow and IOP.

It is well established that aqueous humor levels of TGF β 2 are elevated in primary open-angle glaucoma (POAG) patients (Inatani et al., 2001; Ochiai and Ochiai, 2002; Ozcan et al., 2004; Tripathi et al., 1994). We and others have shown that TGF β 2 treatment of TM cells alters the ECM composition (Fleenor et al., 2006; Fuchshofer et al., 2007; Wordinger et al., 2007) and induces ECM cross-linking (Sethi et al., 2011; Tovar-Vidales et al., 2011; Welge-Lussen et al., 1999). The addition of TGF β 2 elevates IOP in the anterior segment perfusion organ culture models (Fleenor et al., 2006; Gottanka et al., 2004), and over-expression of a bioactivated form of TGF β 2 in mouse eyes causes ocular hypertension (Shepard et al., 2010). TGF β 2 is known to regulate the expression of ECM proteins through the canonical Smad pathway as well as non-canonical signaling pathways (Javelaud and Mauviel, 2004a, b, 2005; Massague and Chen, 2000). We have previously demonstrated that TGF β 2 signals through the canonical Smad and non-Smad pathways and alters the ECM in both human TM cells and in human optic nerve head cells (Sethi et al., 2011; Tovar-Vidales et al., 2011; Zode et al., 2011). Here, we demonstrate that TGF β 2 signaling through the canonical Smad pathway is essential for TGF β 2-induced ocular hypertension in mice.

Our model system utilizes an adenovirus 5 (Ad5) viral vector to over-express a bioactivated form of human TGF β 2 in the TM of mouse eyes. We have previously reported the preparation and use of the Ad5 vector, Ad5.hTGF β 2^{226/228} (Shepard et al., 2010). The Ad5 vector was prepared by the Gene Transfer Core Facility, University of Iowa, Iowa City, IA. Ad5.hTGF β 2^{226/228} (2.5×10^7 pfu) was intravitreally injected in one eye of each animal and the contralateral uninjected eyes were used as negative controls. Previously we have shown that intravitreal injections of Ad5 transgenes produce a more pronounced and consistent IOP response compared to intracameral injections in mouse eyes (McDowell et al., 2012; Millar et al., 2008; Shepard et al., 2007; Shepard et al., 2010). We have also demonstrated that Ad5.null, Ad5.GFP, and transgenes not associated with glaucomatous phenotypes have no effect on ocular hypertension (McDowell et al., 2012; Millar et al., 2008; Shepard et al., 2007; Shepard et al., 2010). We also confirmed that the Ad5.GFP virus has a similar tropism to the TM in each strain used in our current experiments.

In order to test whether TGF β 2 signals through the canonical Smad signaling pathway in the TM to induce ocular hypertension in our *in vivo* mouse model, we utilized 129-Smad3^{tm1Par/J} mice. 129-Smad3^{tm1Par/J} mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred and aged at the University of North Texas Health Science Center (UNTHSC). We utilized homozygous wildtype (WT), heterozygous (HET), and homozygous knockout (KO) 129-Smad3^{tm1Par/J} mice in each of our experiments to test the influence of Smad3 on TGF β 2 signaling and ocular hypertension. Both male and female mice were used, and all mice were 5–6 months old at the start of the experiments unless otherwise noted. All experiments were conducted in compliance with the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research and the UNTHSC Animal Care and Use Committee regulations.

IOP was measured as previously described (Kim et al., 2007). Mice were anesthetized with 2.5% isoflurane + 100% oxygen and IOP was measured with a rebound tonometer (TonoLab; Colonial Medical Supply, Franconia, NH). All measurements were made during

the same 3-hour period of the lights-on phase. This study included IOP measurements from 9 WT mice, 9 HET mice, and 10 KO mice, measured 1–2 times a week for 5 weeks. Area under the curve (AUC) was calculated for each individual mouse and then averaged for each mouse strain. The IOP exposure was calculated by subtracting the AUC of uninjected control eyes from the AUC of the Ad5.hTGF β 2^{226/228} injected eyes. Statistical significance was calculated by one-way ANOVA and tukey *post-hoc* analysis. All data is reported as mean $\pm$ SEM.

At the end of the 5 week time course, whole eyes were removed and processed for immunohistochemistry to detect fibronectin (FN) expression (WT n=5, HET n=4, KO n=7). Eyes were fixed in 4% PFA for 24 hours, processed and embedded in paraffin. 5- μ m sections were cut and sections were transferred to glass slides. Paraffin sections were dewaxed 2 times in xylene, 100% ethanol, and 95% ethanol for 2 minutes each. Slides were then soaked in PBS for 5 minutes. Rabbit anti-fibronectin antibody (Catalog number AB1945, EMD Millipore, Billerica, MA) was used at a 1:1000 dilution, followed by biotinylated secondary anti-goat antibody. Direct ABC immunohistochemistry (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) was performed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB chromogen, DAKO, Carpinteria, CA) as the substrate and is visible as brown in the images of mouse anterior segments. Hematoxylin and Eosin (H&E) staining was performed on additional sections from each eye and is visible as pink and purple stain in the images.

Similarly, whole eyes from each group of mice were collected for qPCR analysis, (WT n=4, HET n=3, KO n=3). The TM rings were carefully dissected from the whole eye. The TM rings contained mainly TM tissue and small amounts of sclera and cornea. Great effort was made to dissect away as much of the sclera and cornea as possible. Samples were homogenized and RNA extracted in Isol-RNA Lysis Reagent (5PRIME, Gaithersburg, MD) and reverse-transcribed to cDNA (Bio-Rad iScript cDNA synthesis Kit; Bio-Rad, Hercules, CA). Each PCR reaction contained: 10 μ l of 2X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 0.25 μ l of forward primer (100 μ M), 0.25 μ l reverse primer (100 μ M), 8.5 μ l dH₂O, and 1.0 μ l of cDNA template (25 ng/ μ l). Primer pairs used in PCR reactions include: FN (5'-GGTGACACTTATGAGCGCCCTA-3', 5'-AACATGTAGCCACCAGTCTCAT-3') and GAPDH (5'-ACTCCACTCACGGCAAATTC-3', 5'-TCTCCATGGTGGTGAAGAACA-3'). PCR conditions were: 95°C for 1 minute and 40X (95°C for 1 minute, 65°C for 45 seconds, 72°C for 45 seconds). Fold change was calculated using the $\Delta\Delta C_t$ method comparing expression to GAPDH and the uninjected control eye. Statistical significance was calculated by one-way ANOVA and tukey *post-hoc* analysis. All data is reported as mean $\pm$ SEM.

In order to test the influence of the canonical Smad signaling pathway in ocular hypertension, we utilized Smad3 WT, HET, and KO mice. Using an Ad5.GFP virus, we confirmed that the Ad5 virus has tropism for the TM in each of the strains and genotypes based on GFP expression at 7 days post-injection (data not shown). These data are similar to previously published results in multiple strains of mice (McDowell et al., 2012; Millar et al., 2008; Shepard et al., 2007; Shepard et al., 2010). We next tested each genotype to determine their susceptibility to TGF β 2 induced ocular hypertension. We evaluated the IOP of each individual mouse 1–2 times a week post injection of Ad5.hTGF β 2^{226/228} for 5 weeks (Figure 1). IOP exposure was significantly different between genotypes (Figure 1A): WT, 187.7 $\pm$ 23.9 mmHg*day (n=9); HET, 95.6 $\pm$ 24.5 mmHg*day (n=9); KO, 52.8 $\pm$ 25.2 mmHg*day (n=10); (one-way ANOVA and tukey post-hoc analysis, p<0.05 WT versus HET, p<0.01 WT versus KO, not significant HET versus KO). WT mice had a significant increase in IOP at 3, 11 and 14 days post-injection, HET mice had a significant increase in IOP at 11 and 14 days post-injection, and KO mice had no significant increase in IOP at any

time point compared to uninjected control eyes (Figure 1B). All data are represented as mean $\pm$ SEM, with one-way ANOVA and tukey post-hoc analysis calculated per each time point. The significance indicated is compared to WT uninjected control eyes. There was no significant difference in IOP between WT, HET, and KO uninjected control eyes at any time point. Since age can have an effect on IOP between strains and genotypes (Savinova et al., 2001), we measured IOP in 3 month old mice of each genotype. There was no statistical difference in IOP between any of the genotypes (mean $\pm$ SEM for WT, 11.6 $\pm$ 1.3 mm Hg (n=8 eyes); HET, 10.7 $\pm$ mmHg M (n=10 eyes); KO, 9.3 $\pm$ 1.2 mmHg (n=8 eyes)). Knockout of Smad3 does not have an effect on baseline IOP. These data suggest that both copies of Smad3 are necessary in order to induce ocular hypertension by TGF β 2 signaling.

In order to test the effect of TGF β 2 signaling on the ECM in the TM, we evaluated the gross morphology of the TM and FN expression in the injected and uninjected eyes of each genotype (Figure 2). H&E staining of paraffin sections revealed similar TM morphology and organization in WT, HET, and KO eyes (Figure 2A–B, 2E–F, 2I–J). Gross anterior chamber morphology was also similar between genotypes (data not shown). Immunohistochemistry was performed to evaluate FN protein expression in the TM and surrounding tissue. The WT Smad3 mice showed increased levels of FN in the TM and surrounding tissue compared to the uninjected control eye, while both HET and KO Smad3 mice maintained similar levels of FN compared to the uninjected control eyes (Figure 2C–D, 2G–H, 2K–L). Since TGF β 2 is a secreted protein, we would expect to see FN expression changes in the TM as well as in the surrounding tissues. In order to quantitate the amount of FN, trabecular meshwork rings were dissected from whole eyes and qPCR was performed on extracted RNA. These results confirm that FN levels are significantly increased ($p < 0.05$) in the WT Smad3 mice (2.23 $\pm$ 0.24 fold) compared to KO Smad3 mice (0.99 $\pm$ 0.19 fold) after injection with the human TGF β 2 transgene (Figure 2M). There was no significant difference in FN expression between the HET and KO Smad3 mice and the WT and HET Smad3 mice, although there was a trend for intermediate FN levels in the HET Smad3 mice (1.50 $\pm$ 0.12 fold). These data indicate that Smad3 is necessary in order to induce FN changes in the trabecular meshwork after injection with Ad5.hTGF β 2^{226/228}.

Increased IOP is a well-established risk factor for developing glaucoma. Aqueous humor outflow resistance is largely dependent on the ECM composition of the TM. Changes in ECM protein deposition can greatly change the ability of aqueous humor to drain properly from the eye. TGF β 2 signaling pathways are known regulators of ECM synthesis and breakdown (Fuchshofer and Tamm, 2012; Verrecchia and Mauviel, 2002). TGF β 2 regulates the expression of ECM proteins through the canonical Smad pathway as well as non-canonical signaling pathways (Javelaud and Mauviel, 2004a, b, 2005; Massague and Chen, 2000; Sethi et al., 2011; Tovar-Vidales et al., 2011). The canonical Smad pathway involves signaling through several Smad receptor proteins, including Smad2 and Smad3 (Massague, 2000; Massague and Chen, 2000). Smad3 has been implicated as being necessary for the fibrotic response associated with TGF β 2 regulation of ECM proteins (Flanders, 2004; Roberts et al., 2003). We have demonstrated that TGF β 2 signals through both the canonical Smad and non-Smad pathways and alters the ECM in TM cells (Sethi et al., 2011; Tovar-Vidales et al., 2011). Here, we tested whether TGF β 2 signaling through the canonical Smad pathway is responsible for TGF β 2-induced ocular hypertension in mice.

Using our established model system, we demonstrated that Smad3 is necessary to induce ocular hypertension and increase TM FN levels in mouse eyes. An Ad5 virus, which has tropism for the TM, containing a bioactivated form of human TGF β 2 was injected into the eyes of WT, HET, and KO Smad3 mice. Only the WT mice had a significant increase in IOP and FN expression. Given that we saw similar IOP, TM morphology, and FN expression in uninjected control eyes in WT, HET, and KO mice, TGF β 2 may also be

signaling through non-Smad pathways as well as the canonical Smad pathway to maintain normal TM function. However, our data suggest that Smad3 is in the canonical Smad pathway responsible for pathologic IOP elevation after TGF β 2 exposure.

References

- Flanders KC. Smad3 as a mediator of the fibrotic response. *Int J Exp Pathol*. 2004; 85:47–64. [PubMed: 15154911]
- Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGF β 2-induced changes in human trabecular meshwork: implications for intraocular pressure. *Invest Ophthalmol Vis Sci*. 2006; 47:226–234. [PubMed: 16384967]
- Fuchshofer R, Tamm ER. The role of TGF- β in the pathogenesis of primary open-angle glaucoma. *Cell Tissue Res*. 2012; 347:279–290. [PubMed: 22101332]
- Fuchshofer R, Yu AH, Welge-Lüssen U, Tamm ER. Bone morphogenetic protein-7 is an antagonist of transforming growth factor- β 2 in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2007; 48:715–726. [PubMed: 17251470]
- Gottanka J, Chan D, Eichhorn M, Lutjen-Drecoll E, Ethier CR. Effects of TGF- β 2 in perfused human eyes. *Invest Ophthalmol Vis Sci*. 2004; 45:153–158. [PubMed: 14691167]
- Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor- β 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch Clin Exp Ophthalmol*. 2001; 239:109–113. [PubMed: 11372538]
- Javelaud D, Mauviel A. Mammalian transforming growth factor- β s: Smad signaling and physiopathological roles. *Int J Biochem Cell Biol*. 2004a; 36:1161–1165. [PubMed: 15109563]
- Javelaud D, Mauviel A. Transforming growth factor- β s: smad signaling and roles in physiopathology. *Pathol Biol (Paris)*. 2004b; 52:50–54. [PubMed: 14761714]
- Javelaud D, Mauviel A. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF- β : implications for carcinogenesis. *Oncogene*. 2005; 24:5742–5750. [PubMed: 16123807]
- Kim CY, Kuehn MH, Anderson MG, Kwon YH. Intraocular pressure measurement in mice: a comparison between Goldmann and rebound tonometry. *Eye (Lond)*. 2007; 21:1202–1209. [PubMed: 16946746]
- Lutjen-Drecoll E. Functional morphology of the trabecular meshwork in primate eyes. *Prog Retin Eye Res*. 1999; 18:91–119. [PubMed: 9920500]
- Massague J. How cells read TGF- β signals. *Nat Rev Mol Cell Biol*. 2000; 1:169–178. [PubMed: 11252892]
- Massague J, Chen YG. Controlling TGF- β signaling. *Genes Dev*. 2000; 14:627–644. [PubMed: 10733523]
- McDowell CM, Luan T, Zhang Z, Putliwala T, Wordinger RJ, Millar JC, John SW, Pang IH, Clark AF. Mutant human myocilin induces strain specific differences in ocular hypertension and optic nerve damage in mice. *Exp Eye Res*. 2012; 100:65–72. [PubMed: 22575566]
- Millar JC, Pang IH, Wang WH, Wang Y, Clark AF. Effect of immunomodulation with anti-CD40L antibody on adenoviral-mediated transgene expression in mouse anterior segment. *Mol Vis*. 2008; 14:10–19. [PubMed: 18246028]
- Ochiai Y, Ochiai H. Higher concentration of transforming growth factor- β in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn J Ophthalmol*. 2002; 46:249–253. [PubMed: 12063033]
- Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF- β 2 in patients with glaucoma. *Int Ophthalmol*. 2004; 25:19–22. [PubMed: 15085971]
- Quigley HA. Open-angle glaucoma. *N Engl J Med*. 1993; 328:1097–1106. [PubMed: 8455668]
- Roberts AB, Russo A, Felici A, Flanders KC. Smad3: a key player in pathogenetic mechanisms dependent on TGF- β . *Ann N Y Acad Sci*. 2003; 995:1–10. [PubMed: 12814934]
- Rohen JW, Witmer R. Electron microscopic studies on the trabecular meshwork in glaucoma simplex. *Albrecht Von Graefes Arch Klin Exp Ophthalmol*. 1972; 183:251–266. [PubMed: 4111808]

- Savinova OV, Sugiyama F, Martin JE, Tomarev SI, Paigen BJ, Smith RS, John SW. Intraocular pressure in genetically distinct mice: an update and strain survey. *BMC Genet.* 2001; 2:12. [PubMed: 11532192]
- Sethi A, Jain A, Zode GS, Wordinger RJ, Clark AF. Role of TGFbeta/Smad signaling in gremlin induction of human trabecular meshwork extracellular matrix proteins. *Invest Ophthalmol Vis Sci.* 2011; 52:5251–5259. [PubMed: 21642622]
- Shepard AR, Jacobson N, Millar JC, Pang IH, Steely HT, Searby CC, Sheffield VC, Stone EM, Clark AF. Glaucoma-causing myocilin mutants require the Peroxisomal targeting signal-1 receptor (PTS1R) to elevate intraocular pressure. *Hum Mol Genet.* 2007; 16:609–617. [PubMed: 17317787]
- Shepard AR, Millar JC, Pang IH, Jacobson N, Wang WH, Clark AF. Adenoviral gene transfer of active human transforming growth factor- β 2 elevates intraocular pressure and reduces outflow facility in rodent eyes. *Invest Ophthalmol Vis Sci.* 2010; 51:2067–2076. [PubMed: 19959644]
- Tovar-Vidales T, Clark AF, Wordinger RJ. Transforming growth factor-beta2 utilizes the canonical Smad-signaling pathway to regulate tissue transglutaminase expression in human trabecular meshwork cells. *Exp Eye Res.* 2011; 93:442–451. [PubMed: 21722634]
- Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp Eye Res.* 1994; 59:723–727. [PubMed: 7698265]
- Verrecchia F, Mauviel A. Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. *J Invest Dermatol.* 2002; 118:211–215. [PubMed: 11841535]
- Welge-Lussen U, May CA, Eichhorn M, Bloemendal H, Lutjen-Drecoll E. AlphaB-crystallin in the trabecular meshwork is inducible by transforming growth factor-beta. *Invest Ophthalmol Vis Sci.* 1999; 40:2235–2241. [PubMed: 10476788]
- Wordinger RJ, Fleenor DL, Hellberg PE, Pang IH, Tovar TO, Zode GS, Fuller JA, Clark AF. Effects of TGF-beta2, BMP-4, and gremlin in the trabecular meshwork: implications for glaucoma. *Invest Ophthalmol Vis Sci.* 2007; 48:1191–1200. [PubMed: 17325163]
- Zode GS, Sethi A, Brun-Zinkernagel AM, Chang IF, Clark AF, Wordinger RJ. Transforming growth factor-beta2 increases extracellular matrix proteins in optic nerve head cells via activation of the Smad signaling pathway. *Mol Vis.* 2011; 17:1745–1758. [PubMed: 21738403]

Figure 1. Smad3 is necessary for TGFβ2-induced ocular hypertension

All mice were injected in one eye with 2.5×10^7 pfu Ad5.hTGFβ2^{226/228} and IOP was measured for 35 days post injection. The contralateral uninjected eye served as a control. (A) IOP exposure was significantly different between genotypes: WT, 187.7 ± 23.9 mmHg*day (n=9); HET, 95.6 ± 24.5 mmHg*day (n=9); KO, 52.8 ± 25.2 mmHg*day (n=10); (one-way ANOVA, $p < 0.05$ WT versus HET, $p < 0.01$ WT versus KO, not significant HET versus KO). (B) WT mice had a significant increase in IOP at 3, 11, and 14 days post-injection, HET mice had a significant increase in IOP at 11 and 14 days post-injection, and KO mice had no significant increase in IOP at any time point compared to uninjected control eyes (one-way ANOVA, * $p < 0.05$, ** $p < 0.01$). Data reported as mean ± SEM.

Figure 2. Smad3 is necessary for TGFβ2-induced FN expression in the TM

All mice were injected in one eye with 2.5×10^7 pfu Ad5.hTGFβ^{226/228}, and tissue was harvested 35 days post injection. The contralateral uninjected eye served as a control. (A–B, E–F, I–J) H&E staining revealed similar TM morphology and organization in WT, HET, and KO eyes, red * indicates TM. (C, G, K) WT, HET, and KO Smad3 mice all showed similar levels of basal FN expression in uninjected control eyes. (D) WT Ad5.hTGFβ^{226/228} injected eyes showed a pronounced increase in FN expression. (H, L) HET and KO Ad5.hTGFβ^{226/228} injected eyes had similar FN expression as the uninjected control eyes. (M) By qPCR analysis FN levels are significantly increased in the WT mice (2.23 ± 0.24 fold) compared to KO mice (0.99 ± 0.19 fold). There was no significant difference in FN expression between the HET mice (1.50 ± 0.12 fold) and either WT or KO mice. Data reported as mean $\pm$ SEM.