FOXC1 Regulates Expression of Prostaglandin Receptors Leading to an Attenuated Response to Latanoprost

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Submitted: October 24, 2017
Accepted: April 13, 2018
Citation: Doucette LP, Footz T, Walter MA. FOXC1 regulates expression of prostaglandin receptors leading to an attenuated response to latanoprost. Invest Ophthalmol Vis Sci. 2018;59:2548–2554. https://doi.org/10.1167/iovs.17-23223

PURPOSE. This study examines the effect of FOXC1 on the prostaglandin pathway in order to explore FOXC1’s role in the prostaglandin-resistant glaucoma phenotype commonly seen in Axenfeld-Rieger syndrome.

METHODS. Binding and transcriptional activity of FOXC1 to the gene coding for the EP3 prostaglandin receptor (PTGER3) were evaluated through ChIP-qPCR and luciferase-based assays. Immortalized trabecular meshwork cells (TM1) and HeLa cells had FOXC1 mRNA reduced via siRNA interference. qPCR and Western blot experiments were conducted to examine the changes in prostaglandin receptor expression brought about by lowered FOXC1. TM1 cells were then treated with 10 μM latanoprost acid and/or an siRNA for FOXC1. The expression of fibronectin and matrix metalloproteinase 9 were evaluated via qPCR in each treatment condition.

RESULTS. ChIP-qPCR and luciferase experiments confirmed that FOXC1 binds to and activates transcription of the EP3 gene prostaglandin receptor. qPCR and Western experiments in HeLa and TM1 cells showed that FOXC1 siRNA knockdown results in significantly lowered EP3 levels (protein and RNA). In addition, RNA levels of the other prostaglandin receptor genes EP1 (PTGER1), EP2 (PTGER2), EP4 (PTGER4), and FP (PTGER) were altered when FOXC1 was knocked down in TM1 and HeLa cells. Analysis of fibronectin expression in TM1 cells after treatment with 10 μM latanoprost acid showed a statistically significant increase in expression; this increase was abrogated by cotreatment with a siRNA for FOXC1.

CONCLUSIONS. We show the abrogation of latanoprost signalling when FOXC1 is knocked down via siRNA in a trabecular meshwork cell line. We propose that the lower levels of active FOXC1 in Axenfeld-Rieger syndrome patients with glaucoma account for the lack of response to prostaglandin-based medications.

Keywords: prostaglandins, Glaucoma, latanoprost, Axenfeld-Rieger, FOXC1

Glaucoma is a leading cause of blindness in developed nations and is caused by the death of retinal ganglion cells within the retina. It has been predicted that 79.6 million people will be affected by glaucoma by the year 2020, 10% of whom will be bilaterally blind.1 Risk factors for the development of glaucoma include corticosteroid use, smoking, age, and ethnicity.2 However, the major risk factor for glaucoma is an increase in intraocular pressure (IOP). Generation and maintenance of IOP occurs through aqueous humor (AH) flow. Outflow of AH occurs through two outflow facilities, conventional and uveoscleral, generating this IOP, which maintains the globular structure of the eye. As increased IOP is the major risk factor for developing glaucoma, it is also the primary target for medical intervention. To manage IOP, surgical or pharmaceutical interventions are employed to either increase AH outflow or to decrease the amount of AH produced. The most common treatment for glaucoma is the administration of prostaglandin-based medications to lower IOP through increasing flow via the uveoscleral outflow facility. This outflow occurs through the supraciliary space in the ciliary muscle and accounts for approximately 5% to 10% of AH outflow.3

Prostaglandins are proinflammatory molecules derived from arachidonic acid of the phospholipid membrane. They have been shown to be involved in pain response,1 inflammatory responses, bone growth,5 and cilia formation and function.6 In the context of glaucoma, prostaglandin F2 (PGF2) analogs such as latanoprost, bimatoprost, travoprost, and tafluprost are the frontline medications used to lower IOP in glaucomatous patients. Previous studies of these drugs (particularly latanoprost and bimatoprost) have shown that they increase AH outflow by increasing lymphatic drainage through the ciliary muscle, which can lead to long-term IOP reduction.7 Axenfeld-Rieger syndrome (ARS) is an autosomal dominant condition associated with ocular manifestations including iris hypoplasia, displaced Schwalbe’s line, and corectopia.8 Approximately 50% of ARS patients develop open-angle glaucoma. Extraocular manifestations may include craniofacial malformations, mandibular hypoplasia, periumbilical skin, and rarely cardiac/renal malformations. Mutations in one of two transcription factors (FOXC1 or PITX2) are known to cause ARS through deletions, duplications (FOXC1), missense, or nonsense mutations. A previous study in our lab aimed to elucidate the phenotypes involved in ARS and indicated that ARS patients with glaucoma do not respond to conventional therapies aimed to lower IOP.9 These therapies include prostaglandin analogs, β-blockers, carbonic anhydrase inhibitors, γ-agonists, and surgical interventions such as trabeculectomy. A second study performed in our lab used nickel-agarose chromatin enrichment to...
identify downstream targets of FOXC1. Through nickel-agarose chromatin enrichment, we identified the prostaglandin E2 receptor class 3 (EP3) as a potential target for FOXC1 transcription.10 EP3 (coded by PTGER3), is one of four PGE2 G-protein coupled receptors (GPCR) along with EP1, EP2, EP4. The majority of glaucoma medications (including latanoprost, bimatoprost, and travoprost) are based on PGE2. These drugs bind to the receptor FP, which has been previously shown to have complicated cross-talk with the other classes of prostaglandin GPCR (for full review of prostaglandins in glaucoma, see Doucette and Walter1). In this study, we show that FOXC1 can directly manipulate the prostaglandin signaling pathway by transcriptional regulation of the receptors EP1, EP2, EP3, EP4, and FP. We also show the abrogation of latanoprost signaling when FOXC1 is knocked down via siRNA, when FOXC1 is knocked down via siRNA in a trabecular meshwork cell line. We propose that the lower levels of active can directly manipulate the prostaglandin signaling pathway by transcriptional regulation of the receptors EP1, EP2, EP3, EP4, and FP. We also show the abrogation of latanoprost signaling when FOXC1 is knocked down via siRNA, when FOXC1 is knocked down via siRNA in a trabecular meshwork cell line. We propose that the lower levels of active

**Materials and Methods**

**Cell Culture and Transfection**

TM1 cells were obtained from Polansky et al.11 and HeLa cells were obtained from ATCC (ATCC CCL-2). All cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies Inc., Burlington, ON, Canada) and supplemented with 10% fetal bovine serum. siRNAs were transfected using Lipofectamine 2000 (Life Technologies Inc.) according to the manufacturer’s instructions. The cells were transfected for 24 to 48 hours before harvesting.

**Chromatin Immunoprecipitation and Quantification**

HeLa cells were grown to confluency and chromatin immunoprecipitation was carried out as previously described.12 Briefly, confluent HeLa cells were fixed and their nuclei were lysed. Chromatin was incubated with 5 μg of either rabbit anti-IgG (CALTAG Laboratories, Buckingham, UK), a negative control; rabbit anti-H3AcK9 (Enzo Life Sciences, Farmingdale, NY, USA), a positive control for transcriptionally active chromatin; or goat anti-FOXC1 (OriGene, Rockville, MD, USA). Agarose protein A/G beads were added to the lysates and incubated overnight. The fold enrichment qPCR method (primer efficiency to the power of ΔCt [C_target – C_smc]) was used to determine enrichment of the PTGER fragment (hg38Chr1:g.71430992-g.71430915; Fig. 1A) upon immunoprecipitation with the aforementioned FOXC1 antibodies. Primer efficiencies were calculated using the standard curve method as previously described.12 Enrichment values were tested for significance using an unpaired, one-tailed Student’s t-test.

**Transactivation Assay**

A luciferase-containing vector (pGL3) was modified with a thymidine kinase (TK) basal promoter sequence as previously described12 (herein referred to as pGL3-TK) to test for the presence of an enhancer element. The fragment from PTGER3 identified previously10 was cloned downstream of pGL3-TK (pGL3-TK-PTGER3). A pGL3-TK reporter with a synthetic 6x FOXC1 binding site was used as a positive control. A total of 500 ng of plasmids expressing WT FOXC1 (pcDNA4-Xpress-FOXC1[WT]) or FOXC1 p.S131L (pcDNA4-Xpress-FOXC1[p.S131L]) were transfected as noted previously into HeLa cells along with 60 ng of the tested plasmid (pGL3-TK, pGL3-TK-PTGER3), and 30 ng of pCMV-β-galactosidase as a normalizing transfection control. The cells were harvested using reporter lysis buffer (Promega, Madison, WI, USA), and transactivation assays were carried out per the supplier’s recommendations. Each experiment was conducted in triplicate and performed three times. Luciferase values were normalized to the β-galactosidase controls. Normalized values were analyzed using an unpaired, one-tailed Student’s t-test.

**FOXC1 Binding Site Predictions**

A matrix of FOXC1 binding sites was used in conjunction with POSSUM software (available in the public domain, accessed April 20, 2018, https://zlab.bu.edu/~mfrith/possum/) to predict putative binding of FOXC1 to the genomic regions of PTGER1, PTGER2, PTGER3, PTGER4, and PTGFR including 2000 bp upstream of the 5’ UTR. Default parameters were used for each analysis (score threshold 5, residue abundance range 100, pseudocount 0.375).

**Western Blot and Protein Analysis**

Western analysis was performed as previously described.12 In brief, TM1 and HeLa cells were transfected with an siRNA for FOXC1 or a control scrambled siRNA. Cell lysates were harvested and electrophoretically separated on a polyacrylamide gel and then transferred to a nitrocellulose membrane. The membranes were probed using rabbit anti-FOXC1 (1:1000; OriGene), rabbit anti-EP3 (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-α-tubulin (1:10,000; Santa Cruz Biotechnology, Rockford, IL, USA). The net intensity of bands was normalized to the net intensity of α-tubulin and then scaled to the control for each treatment. Experiments were repeated at least in triplicate.

**RNA Extraction and Real-Time Quantitative PCR**

Total RNA was extracted from transfected HeLa and TM1 cells using TriZol reagent (ThermoFisher Scientific, Rockford, IL, USA) per the manufacturer’s instructions. RNA quantity and quality was assessed using the 260/280 method. One microgram of RNA was converted to cDNA using a MMLV reverse transcriptase reaction. The QuantiTect SYBR green PCR assay (Qiagen, Toronto, ON, Canada) was used to calculate relative quantities of PTGER1, PTGER2, PTGER3, PTGER4, PTGFR, matrix metalloproteinase 9 (MMP9), fibronectin, FOXC1, and HPRT1 (endogenous control) RNA on an ABI 7900HT (Life Technologies). FOXC1 mRNA was quantified to verify FOXC1 knockdown. Samples transfected with scrambled control siRNA were compared with those transfected with siRNA-FOXC1. Each experiment was performed in triplicate a minimum of three times. Sample wells were excluded if the Ct value was >0.5 from the median of the triplicate. If more than one sample well was rejected, the dataset was excluded from further analysis. Primer efficiencies were calculated using a standard curve method. The ΔΔ Ct method was used to calculate the relative quantities of RNA in comparison to the siRNA scrambled control as previously described.12 An unpaired, two-tailed Student's t-test was used to calculate statistical significance.
Latanoprost Treatment

Latanoprost acid (Toronto Research Chemical, North York, Ontario, Canada) was dissolved in 95% EtOH at a stock concentration of 100 mg/mL. TM1 cells (2.0 × 10^5) were seeded on six-well dishes and allowed to grow as noted above for 24 hours. Cells were transfected as noted previously with a scrambled siRNA control, or an siRNA for human FOXC1. After 24 hours of transfection, latanoprost or an equivalent volume of 95% EtOH (vehicle) was diluted in Opti-MEM media (ThermoFisher Scientific, Rockford, IL, USA) and added to TM1 cells at a 10 μM final concentration for 24 hours. qPCR analysis was carried out on MMP9 and fibronectin as noted previously. Primers were designed as per Li et al. 13 Relative quantities were normalized to HPRT1 (housekeeping gene) and scaled to the scrambled siRNA treated with the vehicle control. Experiments were repeated five times in triplicate. An unpaired, two-tailed Student’s t-test was used to calculate statistical significance.

RESULTS

FOXC1 Binding and Transcriptional Assays

Primers surrounding the previously discovered PTGER3 fragment10 were used to amplify the fragment from immuno-precipitated chromatin. A goat-anti FOXC1 antibody showed enhanced precipitation of the PTGER3 fragment in comparison to the IgG negative control fragment upon agarose gel electrophoresis (data not shown). This was confirmed using qPCR, which showed a 3.3-fold enrichment in comparison to the IgG negative control (Fig. 1A). Relative quantities were normalized to HPRT1 (housekeeping gene) and scaled to the scrambled siRNA treated with the vehicle control. Experiments were repeated five times in triplicate. An unpaired, two-tailed Student’s t-test was used to calculate statistical significance.

FOXC1 in a Prostaglandin-Resistant Glaucoma Phenotype

IOVS | May 2018 | Vol. 59 | No. 6 | 2550

FIGURE 1. (A) Position of the PTGER3 fragment identified by Tamimi et al.10 in intron 3 of the PTGER3 gene (left). ChIP-qPCR results showing enhanced binding of FOXC1 to the PTGER3 fragment of approximately threefold. H3AcK9 was used a control for euchromatin (right). (B) Summary of results from luciferase-based experiments. The PTGER3 fragment was cloned downstream of a thymidine kinase promoter and upstream of luciferase. The presence of exogenous wildtype (WT) FOXC1 causes an increased luciferase signal from the PTGER3 fragment when compared with a mutant (p.S131L) that abolishes FOXC1’s binding ability.

Luciferase reporter construct with an upstream TK promoter (pGL3-TK-PTGER3). Transactivation experiments in HeLa cells transfected with a pcDNA4-Xpress-FOXC1(WT) construct showed a 50% increase in luciferase intensity compared to an empty expression vector (pcDNA4-Xpress-Empty). No increase in luciferase activity was seen when the cells were transfected with pcDNA4-Xpress-FOXC1(p.S131L), which expresses FOXC1 with a patient mutation known to cause a lack of transactivation.14 POSSUM analysis of the genomic regions of the prostaglandin receptor genes PTGER1, PTGER2, PTGER3, PTGER4, and PTGFR showed that FOXC1 has one potential binding site upstream of PTGER1, and two upstream of PTGER2, although all these sites are just above the score threshold of 5 (Supplementary Table S1). Interestingly, there are a number of potential binding sites both upstream and within the genes of PTGER3, PTGER4, and PTGFR (Supplementary Table S1).

Protein and RNA Analysis of Prostaglandin Receptors

As we identified binding and transactivation of FOXC1 from the PTGER3 gene, we then examined the effect of reduced FOXC1 levels on EP3 protein levels in HeLa and TM1 cells via Western analysis. Quantification of the EP3 protein after FOXC1 was reduced via siRNA transfection showed an average reduction of 38% in HeLa cells (P = 0.048) and 29% in TM1 cells (P = 0.009; Fig. 2A). We then evaluated the effect of FOXC1 knockdown on the mRNA levels using qPCR analysis. RNA was obtained from HeLa and TM1 cells treated with either a scrambled siRNA control or an siRNA for FOXC1. Primers were designed to amplify each of PGE2 receptor genes, PTGER1 (EP1), PTGER2 (EP2), PTGER3 (EP3), and PTGER4 (EP4) and the PGF2 receptor gene PTGFR (FP) via q-RT-PCR. Primer sequences are available upon request. qPCR analysis in HeLa cells showed a significant reduction in expression of all five analyzed receptor
Figure 2. (A) Representative Western blot analysis of EP3 protein upon knockdown of FOXC1 via siRNA (left). Summary of results from quantification of normalized (EP3 protein intensity/α-tubulin protein intensity) EP3 protein from Western experiments in HeLa and TM1 cells (right). (B) qPCR results in HeLa and TM1 cells of PTGER1, PTGER2, PTGER3, PTGER4, and PTGFR upon knockdown of FOXC1. Error bars are expressed as the standard error of the mean; *P < 0.05, **P < 0.01.

Latanoprost Treatments

We used primers to amplify MMP9, fibronectin, and NFkBp65 as per Li et al. to examine the effects of latanoprost on TM1 cells transfected with siRNA for FOXC1. When TM1 cells were treated with 10 μM latanoprost for 24 hours and a control scrambled siRNA, we observed a statistically significant increase in the expression of fibronectin by 1.62-fold (P = 0.006) compared to the scrambled siRNA + vehicle control. We also observed a 1.87-fold increase (P = 0.047) in the expression of fibronectin when FOXC1 was knocked down in the absence of latanoprost. When FOXC1 was lowered via siRNA and 10 μM latanoprost was added, there was no statistically significant change in fibronectin expression (P = 0.155). MMP9 showed a 2.4-fold increase (P = 0.006) in RNA upon knockdown of FOXC1, although no statistically significant increase was shown with latanoprost (P = 0.08). A statistically significant increase in MMP9 mRNA was detected when FOXC1 was knocked down, and the cells were treated with 10 μM latanoprost acid (P = 0.05). Results are summarized in Figure 3. We also examined the effects of NFkBp65 as per Li et al. but we observed no statistically significant differences under any of the described conditions (data not shown).

Discussion

Clinically, PGF2 analogs such as latanoprost, bimatoprost, or travoprost are considered the first choice for lowering of IOP. The administration of these drugs can cause the relaxation of the trabecular meshwork; however, the major effects of these medications are through relaxation of the ciliary muscle, increasing uveoscleral outflow. Neverthe-
specific effects of FOXC1 on the PGE2 receptors via qPCR. When FOXC1 expression was lowered via siRNA, we observed reduced expression of PTGER1, PTGER2, and PTGER3 in both TM1 and HeLa cell lines; however, PTGER4 and PTGFR were upregulated in TM1 cells, but lowered in HeLa cells. To investigate the potential for direct regulation of these receptors via FOXC1, in silico analysis was performed using the POSSUM prediction program and a FOXC1 binding site matrix to predict putative binding sites of FOXC1 in the genomic region of each gene. These predictive analyses indicate FOXC1 binding potential at one and two sites within the genes PTGER1 and PTGER2, respectively. Of interest, there appear to be many potential binding sites for FOXC1 in PTGER3, PTGER4, and PTGFR both within 2000 bp of the transcriptional start site and throughout the genomic regions (coding and noncoding) of each gene (Supplementary Table S1). These data suggests that FOXC1 likely plays roles in the expression of these three genes, but may be less important in the regulation of PTGER1 and PTGER2. The limitations of these analyses require further experimentation to determine which of these putative sites truly bind FOXC1. In addition, further sites may be acting further than 2000 bp upstream of each gene and were thus undetected by our analysis. Taken together, our data suggest that although FOXC1 certainly plays a role in prostaglandin receptor regulation and expression, these effects are likely cell/tissue specific (particularly in the cases of PTGER1 and PTGER).

We also show that this dysregulation of prostaglandin receptor expression has consequences on the function of this signaling pathway. Li et al.\textsuperscript{13} showed that the treatment of trabecular meshwork cells with latanoprost acid for 5 days caused the amount of fibronectin present in the extracellular matrix of TM1 cells to increase when examined using immunofluorescence. We showed that the treatment of TM1 cells with 10 μM latanoprost for 24 hours caused a statistically significant 1.62-fold increase in the fibronectin expression via qPCR. Interestingly, we also observed a significant increase of fibronectin expression (1.8-fold) when FOXC1 was knocked down in the presence of vehicle control, suggesting that FOXC1 may play a role in extracellular matrix dynamics through fibronectin expression. When FOXC1 was reduced in TM1 cells in the presence of latanoprost, we observed no statistically significant increase of fibronectin expression. If FOXC1 and latanoprost acid were contributing to fibronectin expression via divergent pathways, we would expect a cumulative increase of fibronectin expression in the siRNA FOXC1-latanoprost experiment. Given that the response is lower than either latanoprost or siRNA FOXC1 alone, we conclude that FOXC1 and latanoprost work through a mutual pathway (Fig. 4). This inhibited response is likely due to the reduction or dysregulation of the prostaglandin signaling pathway as a result of the concomitant dysregulation of receptor transcription. The observation that EP3 is reduced although FP expression is increased in TM1 cells is interesting,
as a study by Ota et al.\textsuperscript{20} showed that the IOP-lowering effects of PGF2 analogs such as tafadroxil were abrogated in mice with a EP3 knockout, showing a vital role for EP3 in the action of these drugs. Hinze et al.\textsuperscript{21} showed that treatment of a nonpigmented ciliary epithelium cell line with latanoprost caused an increase in PGE2. Together this suggests that the production of PGE2 then acts through the EP receptors, particularly through EP3, to bring about the lowering of IOP seen in patients treated with these drugs. With regard to our data, one would expect that an increase in the FP receptor, as was seen in the TM1 cells, would cause an increased response from PGF2 analogs such as latanoprost acid, however, it is most likely that this response in TM1 cells is limited by the lowered EP3 expression. We anticipate that these results are likely similar in all cases of PGF2 analogs as they all act through the FP receptor. In addition, Yu et al.\textsuperscript{22} has shown that treatment of primary trabecular meshwork cells with latanoprost, travoprost, and bimatoprost all increase fibronectin expression. Our data have recapitulated these findings in an immortalized version of these cells. Ultimately, the relationship between FOXC1 and PGF2 analogs is complex and warrants further study within the context of other systems such as primary TM cells or an animal model.

We also show a role of FOXC1 in the expression of MMP9. Li et al.\textsuperscript{13} noted that MOLT-3 cells that express the FP receptor show an increase in MMP9 expression upon treatment with latanoprost acid. However, in our study we noted no statistically significant increase in MMP9 expression in the presence of latanoprost. We did note that MMP9 expression was significantly increased upon knockdown of FOXC1 alone (Fig. 3). We also observed a 2.1-fold change in MMP9 expression seen in the latanoprost acid + FOXC1 siRNA (P = 0.05), although due to the lack of response under exposure to latanoprost, it is most likely that this increase is solely the result of the lowered expression of FOXC1. Along with the change in expression of fibronectin, our data suggest that FOXC1 may have a direct role in tissue/extracellular remodeling within the trabecular meshwork and could regulate the AH dynamics through the conventional outflow pathway as well. That FOXC1 has a role in ECM remodeling is unsurprising considering the vast amount of work done concerning FOXC1 in invasive cancers (reviewed in Elian et al.\textsuperscript{23}). However, our result is contradictory to studies of FOXC1 in nasopharyngeal cancers, as studies show that reduced FOXC1 in nasopharyngeal cancers cells are associated with a decrease in MMP9.\textsuperscript{24,25} These differences are likely due to the use of different cell lines and tissues of origin used in these studies, again suggesting a tissue-specific role of FOXC1 in disease pathogenesis.

This is the first study illustrating a link between FOXC1 and the prostaglandin signaling pathway. Although this study evaluated the role of this interaction in glaucoma, it is possible that this dysregulation of prostaglandin GPCRs can provide some explanation for phenotypes seen in ARS patients. For example, PGE2 has been linked to bone resorption in humans, mice, and osteoclast formation in organ culture,\textsuperscript{26–29} which may have implications in the craniofacial malformations seen in ARS patients.

Although speculative, our data may suggest a mechanism applicable to populations beyond those of ARS patients. Recently, an association study from the NEIGHBORHOOD consortium showed that a SNP located near FOXC1 (rs2745572) is a potential susceptibility locus for primary open angle glaucoma (POAG).\textsuperscript{30} According to dbSNP (available in the public domain, https://www.ncbi.nlm.nih.gov/snp), this SNP has a global minor allele frequency of 36%, and according to Bailey et al.\textsuperscript{31} it has an odds ratio of 1.25 at a P value of 2.56 × 10\textsuperscript{-10} to be associated with POAG. It is possible that patients who have this SNP and develop POAG may have aberrant expression of FOXC1, predisposing these individuals to a recalcitrant form of glaucoma via dysregulation of the prostaglandin pathway. However, the association between the SNP and recalcitrant glaucoma, or between the SNP and aberrant levels of FOXC1, has not been examined. If these associations were proven to be true, the aberration in FOXC1 expression would have to still allow proper development and thus not lead to ARS, but could potentially affect systems in subtler manners, such as POAG.

**Acknowledgments**

Disclosure: L.P. Doucette, None; T. Footz, None; M.A. Walter, None

**References**


