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Optimal procedure for extracting RNA from human ocular tissues and expression profiling of the congenital glaucoma gene *FOXC1* using quantitative RT-PCR

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Purpose: To develop methods for obtaining high quality RNA from human donor eyes and to determine the expression profile of the congenital glaucoma gene *FOXC1* in human ocular tissues.
Methods: To obtain high quality RNA from donor eyes, several different preservation methods were tested including storing eyes on ice, freezing eyes, and placing eyes in the commercial fixative RNA*later*™ prior to dissection and RNA extraction. Nine different ocular tissues from human donors were dissected and examined. Pigment-free total RNA was isolated and used for quantitative real-time RT-PCR using *FOXC1* and GAPDH (internal standard) primers to assess the quality and expression of *FOXC1*.
Results: An expression profile of *FOXC1* in human ocular tissues was determined using quantitative PCR of RNA isolated using a simple and effective procedure for ocular tissue preservation and pigment-free RNA isolation. Higher quality RNA was obtained from human donor eyes preserved in RNA*later*™ compared to RNA extracted from eyes stored on ice or frozen at -80 °C. RNA extraction techniques that removed interfering pigment from ocular tissues produced RNA that could be easily amplified by PCR. In the adult human eye, expression of *FOXC1*was greatest in the trabecular meshwork (TM) followed by the optic nerve head, choroid/RPE, ciliary body, cornea, and iris. *FOXC1* expression levels were much lower in other non-ocular human tissues, such as liver, muscle, lung, heart, and kidney.
Conclusions: Using an optimized donor eye preservation method and tissue RNA isolation procedure, we show that the *FOXC1* transcription factor gene, which is known to be associated with developmental glaucoma, also may have an important role in the adult eye.

Glaucoma is a leading cause of irreversible blindness in the world [1], and consists of a heterogeneous group of optic neuropathies that share many similar clinical features [2,3]. Damage to the trabecular meshwork (TM) causes the elevated intraocular pressure associated with primary open angle glaucoma, while damage to the optic nerve head (ONH) causes optic disc cupping that is associated with all subtypes of glaucoma. Recent advances in the molecular genetics of glaucoma [4] provide opportunities to better understand the pathogenesis of the various subtypes of glaucoma. The forkhead box C1 gene, *FOXC1*(formerly *FKHL7*), is one of six known glaucoma genes.

Genetic loci responsible for several dominantly inherited anterior segment abnormalities have been mapped on chromosome 6p25 by linkage analysis and identification of mutations in *FOXC1* [5-9]. These ocular disorders include iridogoniodysgenesis anomaly, Axenfeld-Rieger anomaly, familial glaucoma iridogoniodysplasia, and juvenile-onset glaucoma with iridogoniodysgenesis. All are disorders of ocular anterior segment development and are strongly associated with the development of congenital or juvenile glaucoma [4,6-8,10]. Mutations in *FOXC1*, or abnormal regulation of *FOXC1* expression are responsible for most of the glaucoma families that map to 6p25 [5,6,9,11]. *FOXC1* is a member of the winged helix family of transcription factors, and it plays key roles in embryogenesis, tumorigenesis, and regulation of tissue specific gene expression. *FOXC1* is expressed in the developing eye and in a variety of adult tissues [5,6,12,13]. Developmental defects in the anterior segment due to *FOXC1* (*mf1*) in the mouse also have been well described. Heterozygotic *FOXC1* or *FOXC1* null mice develop abnormalities similar to human patients including small or absent Schlemm's canal, aberrantly developed trabecular meshwork, iris hypoplasia, severely eccentric pupils and displaced Schwalbe's line [14].

While many developmental abnormalities of the anterior segment associated with *FOXC1* gene expression have been described in animal models and human patients, the expression pattern of the *FOXC1* gene has not been determined in either animal or human ocular tissues. In this study, we measured the expression level of *FOXC1* in adult human donor ocular tissues to determine if *FOXC1* is selectively expressed in specific tissues associated with glaucoma pathogenesis.

The extraction of RNA from human ocular tissues presents many obstacles. First, RNA isolated from ocular tissues

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is easily degraded. Traditionally, human donor eyes are obtained from an eye bank or hospital and transported to researchers in a moist chamber on ice. There is a requisite delay time for serological testing (e.g., HIV and hepatitis) before the tissues can be handled safely. The post-mortem interval (PMI) between patient death and the subsequent collection of dissected ocular tissues may take from several hours to days. During this time the RNA degrades, thus compromising RNA quality and yield. In addition to RNA stability issues, many ocular tissues contain melanin that co-purifies with RNA and inhibits reverse transcription (RT) as well as the polymerase chain reaction (PCR) [15]. The problem is worse in the heavily pigmented ocular tissues, such as iris [16], ciliary body, and choroid. Finally, some ocular tissues are inherently small (e.g., the trabecular meshwork from a single eye weighs only a few mg in wet weight) so that purification of high quality RNA is a challenge. We assessed relatively simple procedures for obtaining high quality RNA from human eye tissues that are based on improved preservation of donor eyes and pigment-free RNA isolation. Using these procedures, we were able to examine the expression pattern of the *FOXC1* gene in 9 different human ocular tissues.

METHODS

Preservation of donor eyes: Four different methods of preserving donor eyes were tested: (1) storing enucleated eyes on ice in a moist chamber, (2) rapid freezing of whole globes in liquid nitrogen, (3) rapid freezing of whole globes in dry ice, and (4) placing the whole globes in RNA*later*™ (Ambion, Austin, TX) [17]. In each case the right eye (OD) of each donor was either frozen or preserved in RNA*later*™, while the left eye (OS) was kept in a moisture chamber on ice. Rapid freezing of whole globes of normal eyes was performed at the eye bank (The Central Florida Lions Eye and Tissue Bank, Tampa, FL) by placing the eye directly in liquid nitrogen or on dry ice with or without prior removal of the vitreous humor. For RNA*later*™ preservation, eyes were slit posterior to the cornea to remove much of the vitreous and subsequently submerged in approximately 30 ml of RNA*later*™ at the eye bank. Each method was tested with 3 sets of eyes and an average PMI of less than 5 h from the time of death to preservation. Dissected ocular tissues were stored at -80 °C for subsequent RNA isolation.

RNA extraction and cDNA synthesis: RNA was isolated from dissected ocular tissues using commercial kits based on either guanidinium isothiocyanate (GITC) extraction methods (ToTALLY RNA, Ambion, Austin, TX) or silica-based filter-binding methods (RNAqueous-4 PCR, Ambion). In each case, small amounts of tissue (<50 mg) were homogenized in 1 ml of lysis solution using a motorized rotor-stator homogenizer (Brinkmann Instruments, Inc., Westbury, NY). To prevent filter clogging in using silica-based filter-binding methods, the homogenate was repeatedly centrifuged to remove debris and insoluble material before proceeding to filtration. Further extraction steps were performed according to the manufacturer's protocols. RNA quality was assessed by agarose gel electrophoresis. To evaluate the effects of pigment contamination in the RNA, RT-PCR was performed using MultiScribe reverse transcriptase and random hexamers (PE Applied Biosystems, Foster City, CA) and GAPDH primers (5'-ACC ACA GTC CAT GCC ATC AC-3', forward and, 5'-TCC ACC ACC CTG TTG CTG TA-3', reverse) under standard PCR conditions. cDNA from non-ocular human tissues was purchased from BioChain, Inc. (San Leandro, CA). The ocular tissue RNA used for quantitation of *FOXC1*expression was pooled from four normal human eyes (47 year old male, 79 year old male, 73 year old female, and 83 year old female) with <5 h of PMI between death and RNA*later*™ preservation.

Real-time quantitative PCR: Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) according to the manufacturer's instructions. For amplification of *FOXC1*, reactions were performed in 25 µl total volume with 200 nM primers, (5'-TAA GCC CAT GAA TCA GCC G-3', forward and, 5'-GCC GCA CAG TCC CAT CTC T-3', reverse), 100 nM dual labeled probe (FAM-CTT ACC ACG GTG ATG CCT GTG TGC C-TAMRA NHS Ester) and cDNA from 2.5 ng of total RNA in 1X TaqMan universal PCR master mix. The PCR was amplified at 50 °C for 2 min, 95 °C for 10 min and followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. In order to compare expression patterns among tissues, relative quantification of gene expression was performed using the standard curve method according to User Bulletin 2 of the ABI Prism 7700 Sequence Detection System. *FOXC1* quantification data were normalized to expression of the housekeeping gene GAPDH, an endogenous control reagent supplied by PE Applied Biosystems.

RESULTS

Tissue preservation and RNA integrity: RNA from ocular tissues dissected from donor eyes that were preserved in different ways was extracted using the ToTALLY RNA kit, and the RNA quality was checked by agarose gel electrophoresis (Figure 1). Much of the RNA was degraded in the eyes preserved on ice (OS), especially in the TM where the RNA was completely degraded. In contrast, high quality and better yields (about 50% increase) of RNA were obtained from RNA*later*™ preserved tissues (OD), including TM, iris, cornea, ciliary body, retina and choroid/retinal pigment epithelium (Figure 1A). For example, the yield of RNA from RNA*later*™ preserved cornea and TM was 53-58% higher than in the corresponding frozen tissues (Figure 1A). RNA levels were assessed by ethidium bromide staining of RNA gels. Surprisingly, the RNA quality and yields of the dry ice frozen tissues was worse than that from unfrozen eye tissues, as can be seen for the TM, ciliary body and retina (Figure 1B). The use of RNA*later*™ at any point in the collection/dissection process helped to stabilize RNA in eye tissues. For example, the quality of RNA improved when fresh eyes that arrived in moist chambers were placed in RNA*later*™ (with vitreous removed) for a couple of hours before dissection, or when tissues were placed in RNA*later*™ for about 30 min immediately after dissection (data not shown).

Pigment removal from RNA preparations: After establishing an optimal eye preservation method, we studied ways to isolate pigment-free RNA from the dissected ocular tissues. We used commercial RNA isolation kits belonging to two major categories based on: (1) guanidinium isothiocyanate (GITC) extraction methods or (2) silica-based filter-binding methods. We found that the filter-binding method generated pigment-free RNA from pigmented tissues, whereas GITC extraction did not separate the melanin (a visible brown color) from the RNA (Figure 2A). There was no apparent difference between the two systems in overall RNA integrity when assessed by agarose gel electrophoresis (Figure 2B). As a further test of RNA quality, we performed RT-PCR with GAPDH primers. GAPDH was amplified from the pigment-free RNA but not from the melanin contaminated RNA preparations (Figure 2C). Therefore, the silica-based filter method made a significant difference in the quality of RNA obtained from pigmented tissues compared to GITC extraction methods. However, the GITC extraction method provided more than 50% higher RNA yields than filter-binding methods. In these experiments, RNA yields were assessed spectrophotometrically. For example, the yield of RNA per mg of tissue was 0.4 µg from the cornea and 0.54 µg from the optic nerve when using ToTALLY RNA, while only 0.13 µg and 0.25 µg, respectively, when using silica based filter-binding methods. Therefore ToTALLY RNA became the method of choice for isolating RNA from non-pigmented tissues where sample size was limiting (e.g., TM and optic nerve head). Selective use of extraction methods according to tissue type produced RNA of high quality from 9 different human eye tissues including; iris, ciliary body, choroid/RPE, trabecular meshwork, retina, lens, cornea, optic nerve, and optic nerve head in several sets of human donors. Again, total RNA extracted from these tissues was examined first by agarose gel electrophoresis and second by RT-PCR assays using GAPDH primers. No degradation and no pigment contamination/inhibition of RT-PCR was observed with the isolated RNA (Figure 3). We were unable to reverse the melanin inhibition of RT-PCR using bovine serum albumin as reported by others [15] (data not shown).

Ocular FOXC1 *expression:* Expression levels of the glaucoma gene *FOXC1* in various human tissues, including ocular tissues, were determined by real-time PCR. For ocular tissue analyses, total RNA prepared as described above was pooled from four normal, non-diseased human eyes for each tissue analyzed. Non-ocular tissue cDNA was amplified using the same real time PCR procedures with cDNA equivalent to 2.5 ng for each reaction. Two separate analyses were conducted using triplicate cDNA samples from all tissues. The expression profile for *FOXC1* RNA levels relative to GAPDH in human tissues, including different ocular tissues, is shown in Figure 4. The results, presented in order of relative abundance, show higher levels of *FOXC1* gene expression in most ocular

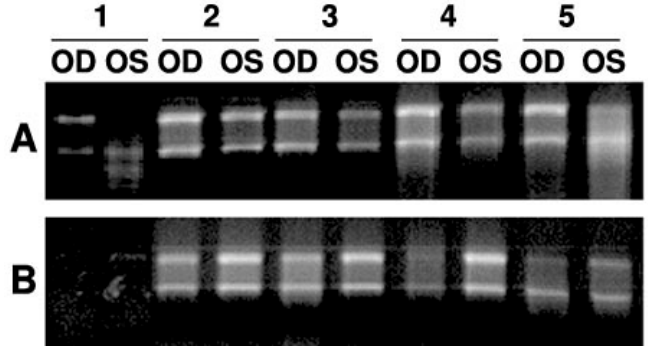

Figure 1. Comparison of RNA integrity from RNA*later*™ preserved or dry-ice frozen tissues. Test eyes (OD) preserved in either 30 ml of RNA*later*™ (**A**) or frozen by dry ice (**B**) were compared to control eyes (OS) from the same donors that were preserved in a moisture chamber on ice. RNA was isolated using Ambion ToTALLY RNA. One µg of total RNA isolated from different tissues was analyzed on a denaturing 1% agarose gel in the presence of ethidium bromide. RNA was examined in the following ocular tissues: trabecular meshwork (lanes 1), iris (lanes 2), cornea (lanes 3), ciliary body (lanes 4), and retina (lanes 5).

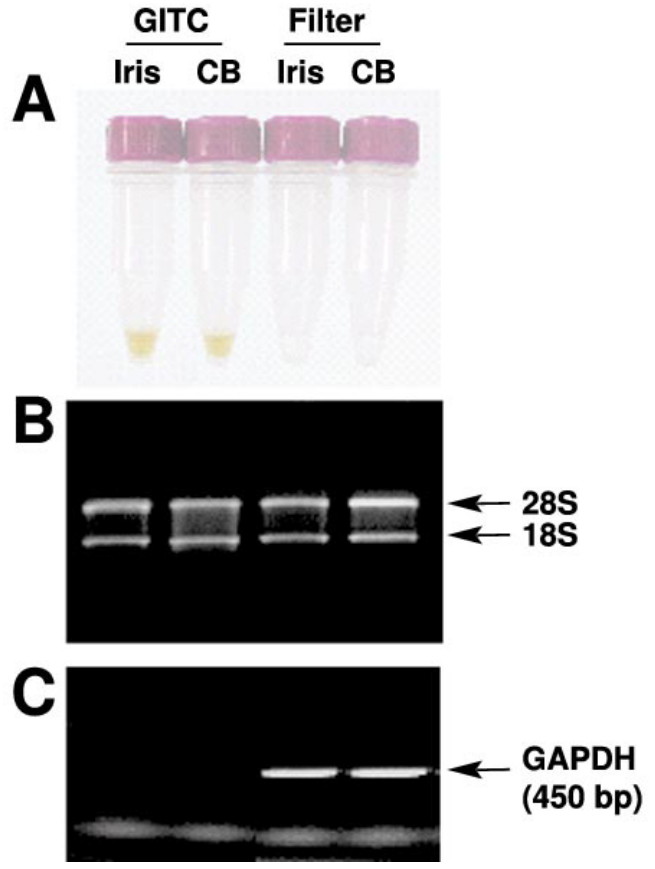

Figure 2. Comparison of RT-PCR amplification of RNA isolated by different methods. The RNA was isolated by either a GITC-based method or a silica-based filter method from pigmented ocular tissues, iris and ciliary body (CB). **A**: Endogenous melanin was observed in the total RNA isolated using the GITC method but not from the filter method. **B**: Total RNA integrity showed no difference between the two methods on a denaturing agarose gel. **C**: Inhibition of RT-PCR in the RNA sample containing endogenous melanin was observed by differential amplification of the house-keeping gene GAPDH.

tissues compared to other non-ocular human tissues. Ocular *FOXC1* expression was most abundant in the trabecular meshwork, followed by the optic nerve head, choroid/RPE, and ciliary body, in that order; the cornea, iris, and optic nerve showed lower expression levels and *FOXC1* was barely detectable in the retina and lens (Figure 4A). Several other human tissues showed comparatively lower levels of *FOXC1* expression, including liver, muscle, lung, heart, pancreas, and brain (Figure 4B). In addition, these quantified *FOXC1* tissue expression levels were confirmed using two other internal standards, another housekeeping gene (β-actin) and the 18S ribosomal gene. The variation of β-actin expression levels among the different tissues (average 1.36±0.97) was larger than the variation for GAPDH (average 1.33±0.65).

DISCUSSION

FOXC1 is an important transcription factor that is involved in the development of ocular anterior segment disorders as well as non-ocular disorders. Chromosomal translocations, deletions, duplications, and nonsense or missense mutations can cause the abnormal expression of *FOXC1*, which has been implicated in the abnormal development of the iris, cornea and TM [6,8,9,11,14,18]. In addition, *FOXC1* also is involved in non-ocular abnormalities including umbilical and dental changes, hydrocephalus, and heart and kidney defects [10,19,20]. Expression of *FOXC1* has been demonstrated in a wide variety of non-ocular tissues [5,6,12] and in whole eyes [6]. However, this study represents the first report of *FOXC1* expression in individual ocular tissues.

Our studies show that expression levels of *FOXC1* were greater in ocular tissues than in the other non-ocular human tissues examined. These results correlate with Northern blot analyses reported by others that showed the eye to be the major *FOXC1* expressing tissue in the mouse [6]. Our studies further show that in the normal adult human eye, *FOXC1* expression is highest in the trabecular meshwork, the tissue known to regulate aqueous outflow and intraocular pressure. Other tissues of the anterior segment such as cornea, iris, and ciliary body also expressed relatively high levels of *FOXC1*. These *FOXC1*expression results are consistent with anterior segment defects found in 6p25 glaucoma patients and in *FOXC1* mutant mice. Localization of the *FOXC1* gene in adult ocular tissues that comprise the aqueous secretory and drainage structures suggests that *FOXC1* may be involved in some way in regulation of aqueous flow in adult human eyes. In addition, *FOXC1* mRNA was expressed at relatively high levels in the posterior segment of the eye in the optic nerve head, a tissue that is associated with the development of glaucomatous optic neuropathy. Although *FOXC1* has traditionally been considered to be an important ocular development gene, it is tempting to speculate that *FOXC1* may play an important role in the adult eye, especially in two of the tissues involved in glaucoma pathogenesis, the trabecular meshwork and optic nerve head.

In our attempts to determine the ocular expression of glaucoma genes and to discover differentially expressed genes associated with various ocular diseases, we encountered serious problems in obtaining high quality RNA from donor eye tissues. To solve this problem, we tested four different methods of preserving donor eyes. We found that freezing eyes in

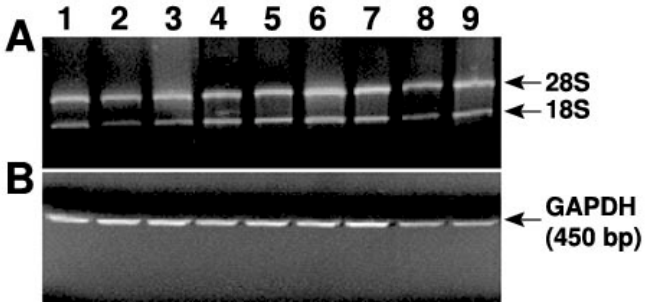

Figure 3. Agarose gel analysis of total RNA and RT-PCR products. **A**: RNA isolated from RNA*later*™ preserved human ocular tissues using either RNAqueous-4PCR (Lanes 1-5) or ToTALLY RNA (Lanes 6-9) on an EtBr-stained denaturing agarose gel. Each lane contains 1 µg of RNA. **B**: RNA quality was determined by RT-PCR amplification of the house-keeping gene GAPDH. RNA examined was from the following ocular tissues: iris (lane 1), ciliary body (lane 2), choroid/RPE (lane 3), trabecular meshwork (lane 4), retina (lane 5), lens (lane 6), cornea (lane 7), optic nerve (lane 8), and optic nerve head (lane 9).

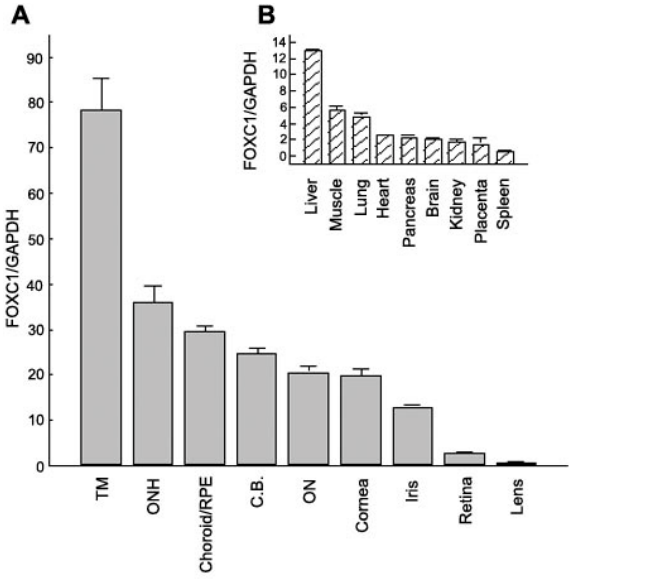

Figure 4. *FOXC1* expression in human tissues. RNA from ocular tissue dissected from four different donor eyes was pooled and reverse transcribed with random primers for quantification by real-time PCR. **A**: *FOXC1* expression (presented in order of relative abundance) in trabecular meshwork (TM), optic nerve head (ONH), choroid/RPE, ciliary body, optic nerve (ON), cornea, iris, retina, and lens was normalized to GAPDH expression levels in the same cDNA samples. Each bar represents the mean ± the standard deviation of 3 replicate analyses. **B** (inset): *FOXC1* expression in non-ocular human tissues by real-time PCR normalized to GAPDH expression levels.

either liquid nitrogen or dry ice resulted in disrupted tissue structure, which made dissection of the thawed preparation more difficult. In addition, rapid freezing of intact eyes in liquid nitrogen often fractured the globe into 2 or 3 pieces resulting in tissue fragmentation and cross-tissue contamination with adjacent tissues. In contrast, RNA*later*™-preserved eyes maintained tissue integrity allowing dissection similar to unpreserved fresh eyes. Based on these results, we conclude that RNA*later*™ tissue storage and stabilization is the preferred method for whole globe preservation. When human donor eyes were preserved in RNA*later*™ by the eye bank immediately after enucleation and before shipment, the PMI was reduced to less than 12 h, cellular RNA was preserved, and eye tissue structure was maintained for easy dissection. An additional advantage of this preservation method is the flexibility it provides in scheduling tissue dissection time without concern for sacrificing RNA quality and quantity.

An additional problem in obtaining high quality RNA from ocular tissues is the significant melanin contamination that is often associated with conventional RNA extraction. Techniques that have been reported by other laboratories to circumvent the problem of melanin interference in the PCR reaction include RNA purification by column chromatography [21], acid-precipitation [22], isolation of mRNA [23], or addition of proteins such as bovine serum albumin or dry milk in the RT-PCR reaction [15,16,24]. However, we found it very difficult to obtain reasonable amounts of melanin-free total RNA from small tissue samples by any of those measures because additional extraction and purification procedures resulted in unacceptable loss of RNA. In addition to the ToTALLY RNA kit, we tested several other GITC-based commercial RNA isolation kits and were unable to produce pigment-free RNA. The filter-binding extraction method, coupled with extensive centrifugation of pigmented homogenates, effectively removed the pigment with minimum reduction in RNA recovery, and made a significant difference in RNA quality.

Using the methods described here, we have successfully established a procedure to preserve human donor eyes and isolate pigment-free RNA from a variety of eye tissues, including heavily pigmented tissues, for RT-PCR analysis. We have collected and dissected over 50 sets of human donor eyes with an interval of up to 12 h between death and preservation and up to 7 days between preservation and dissection. Intact RNA was obtained from trabecular meshwork tissues in every case, whereas previously, we were unable to obtain intact RNA from the trabecular meshwork because of rapid degradation.

Another challenge in quantitatively determining ocular gene expression is the relatively small amounts of extractable RNA from certain ocular tissues, particularly those structures such as the TM and ONH that are involved in glaucoma pathogenesis. Real-time PCR with fluorescence detection is an extremely sensitive and specific assay for quantifying gene expression in small RNA samples [25]. Other quantitative detection methods (e.g., Northern blots, RNase protection assays) require as much as 10 μg of total RNA compared to only 2.5 ng of RNA needed for the real-time PCR analyses. The use of a sensitive quantitative PCR procedure together with improved isolation procedures that produced quality RNA from limited samples of human donor ocular tissues, make it possible for the first time to measure the expression levels of non-abundant RNA such as *FOXC1* in ocular tissues. We found that *FOXC1* expression is highest in the trabecular meshwork and optic nerve head tissues of the adult eye. These tissues are involved in glaucomatous damage to the eye. Therefore, the congenital glaucoma gene *FOXC1* also may be associated with the pathogenesis of adult onset glaucoma. These same techniques will be useful for examining the ocular expression of other glaucoma genes and additional glaucoma candidate genes.

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