Experimental Eye Research 116 (2013) 265–278

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Whole genome expression profiling of normal human fetal and adult ocular tissues

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ARTICLE INFO

Article history:
Received 28 December 2012
Accepted in revised form 13 August 2013
Available online 7 September 2013

Keywords:
gene expression
microarray
retina
retinal pigment epithelium
choroid
sclera
optic nerve
cornea

ABSTRACT

To study growth and development of ocular tissues, gene expression patterns in normal human fetal versus adult eyes were compared. Human retina/retinal pigment epithelium, choroid, sclera, optic nerve* and cornea* tissues were dissected from fetal (24 week gestational age) ($N = 9$; *$N = 6$), and adult ($N = 6$) normal donor eyes. The Illumina® whole genome expression microarray platform was used to assess differential expression. Statistical significance for all comparisons was determined using the Benjamin and Hochberg False Discovery Rate (FDR, 5%). Significant gene expression fold changes > 1.5 were found in adult versus fetal retina/RPE ($N = 1185$), choroid ($N = 6446$), sclera ($N = 1349$), and cornea ($N = 3872$), but not optic nerve. Genes showing differential expression were assessed using Ingenuity Pathway Analysis (IPA) for enriched functions and canonical pathways. In all tissues, development, cell death/growth, cancer functions, and signaling canonical pathways were enriched. There was also a general trend of down-regulation of collagen genes in adult tissues.

1. Introduction

The human visual system is complex and requires numerous tissue and cell types to communicate with each other and the brain throughout the process of development (Kolb et al., 2011). In humans, rapid axial growth of the eye globe is seen in fetal development with slowing toward the end of gestation (Fledelius and Christensen, 1996). However, despite this rapid fetal growth, the human eye is not full sized at birth nor is the visual system fully developed; visual signals and nutritional factors contribute to postnatal ocular development in the early years of life (Bremond-Gignac et al., 2011). During childhood development, an active regulatory process of ocular growth, emmetropization, aims to match the optical power of the cornea and lens to the axial length of the eye (Gordon and Donzis, 1985). Failure of emmetropization in postnatal ocular development commonly results in either myopic or hyperopic refractive error, or blurred vision (Gordon and Donzis, 1985).

Given the impracticality of human sample collection, researchers have utilized animal models to study developmental and late-onset ocular diseases. These animal models have demonstrated that visual cues interpreted by the retina and signaled through the choroid and sclera locally control the shape and size of the eye through unknown mechanisms (Faulkner et al., 2007; Tkatchenko et al., 2009; Wallman and Winawer, 2004; Wildsoet and Wallman, 1995). Human eyes with high degrees of refractive error have distinct clinical phenotypes including changes to their size, shape and tissue structure (Xu et al., 2007). Highly myopic eyes tend to be larger than their emmetropic counterparts, with the elongation primarily localized axially (Atchison et al., 2004), suggesting that changes in any or all of those tissues (central retinal, retinal pigment epithelium [RPE] and sclera) may be responsible for failed emmetropization and myopic development.

A better understanding of the tissue-specific expression differences during normal growth and development is key to identify ocular growth and development mechanisms in human tissues. As collection of postnatal eyes undergoing emmetropization is impractical, fetal ocular tissue types of central retina/RPE, choroid, sclera, optic nerve and cornea were compared to their adult counterparts from normal donor eyes to study patterns

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http://dx.doi.org/10.1016/j.exer.2013.08.009

Table 1
Donor Information for Adult Ocular Whole Globes Used in Tissue Expression Analyses. Preservation time interval (PTI) is listed in hours. Age is listed in years.

Individual	Race	Sex	Age	PTI	Cause of death
2809	Caucasian	Female	76	4:00	Leukemia/acute myeloid leukemia
2835	Caucasian	Female	55	4:55	Dementia
2828	Caucasian	Male	80	5:20	Pneumonia
2834	Caucasian	Male	56	5:22	Abdominal aortic aneurysm rupture/heart disease
2217	Caucasian	Female	77	6:24	Chronic obstructive pulmonary disease/emphysema
2836	Caucasian	Male	67	5:10	Heart disease

of ocular growth during development. Although it is unclear to what extent the mechanisms controlling postnatal emmetropization are active in prenatal development, one gene identified in a study of extreme hyperopic refractive error, *MFRP* (MIM 606227), has been demonstrated to be necessary for both prenatal ocular growth and postnatal emmetropization (Sundin et al., 2008). To our knowledge this is the first whole genome expression analysis comparing human adult versus fetal ocular tissues. This data can provide an understanding of normal changes these tissues undergo during prenatal growth and development in humans and it also may contain clues to understand how diseases such as myopia may result from disruptions to normal growth processes.

2. Materials and methods

2.1. Ocular sample selection

The tissues selected for this study were central retina/RPE, choroid, sclera, optic nerve and cornea. Selection of tissues was based on relevance to disorders studied in our lab such as microphthalmia and myopic exaggerated eye growth, and feasibility of collection. The posterior wall tissues were selected and prioritized for larger sample sizes based on their relation to a focal disease studied in our lab, high myopia. Additionally, the cornea and optic nerve tissues were selected due to their relations with other diseases under study in our lab including glaucoma and corneal abnormalities.

To compare growth and development gene expression in ocular tissue types, normal samples from two age groups were used: fetal eyes and adult eyes. The fetal donor eyes were obtained from Advanced Biosciences Resources (Alameda, CA, USA), while the adult eyes were obtained from the North Carolina Eye Bank (Winston–Salem, North Carolina, USA). Fetal gestational age was determined by most recent menstruation in addition to fetal foot measurements. The group of fetal eyes consisted of late prenatal fetal eyes of approximately 24-weeks gestational age from elective abortions with no known defects or abnormalities. 24-weeks gestational eyes are the oldest prenatal eyes readily available and are undergoing rapid growth and axial elongation (Fledelius and Christensen, 1996). Nine fetal donor eyes (four male and five female samples), and six fully grown adult donor eyes (three of each gender) were used for microarray analyses. Space and cost limitations required that optic nerve and cornea sample sizes were reduced to six adult and six fetal samples each. All adult donors were Caucasian, and donors with known ocular disorders were excluded (Table 1). Ethnic and health information was not available for fetal donors. The study was approved by Duke University's Institutional Review Board and adhered to the tenets of the Declaration of Helsinki guidelines.

2.2. Ocular dissection

All adult whole globes were immersed in RNAlater® (Qiagen, Hilden, Germany) within 6.5 h (Table 1) of collection, and shipped overnight on ice. Several studies have shown that the postmortem delay in preservation of samples within this time frame have limited effects on RNA integrity from brain tissue (Durrenberger et al., 2010; Ervin et al., 2007). Fetal whole globes were collected and preserved in RNAlater® within minutes of collection and shipped overnight on ice. Prior to immersion in RNAlater®, a 2 mm incision was cut equatorially into all whole globes to allow permeation of the solution to the inner tissues while minimizing unwanted physical changes to the tissue, such as retinal tearing. All whole globes were dissected on the same day as arrival. The retina, RPE, choroid and scleral tissues were isolated at the posterior pole using a circular, double embedded technique using round 7 mm and 5 mm biopsy punches. To reduce contamination of retina to the other tissues samples, the second biopsy punch of 5 mm was used in the center of the 7 mm punch after retinal removal. The adult RPE was collected in RNAlater® by gentle brushing from the choroid, pipetting the solution, and centrifuging at 4°C to remove the RNAlater®. Fetal eye samples proved difficult to separate the RPE from the retina. Consequently, the retina and RPE were collected in total. Additionally, optic nerve and corneal samples were isolated from each eye in each age group. Central corneal samples were isolated using a clean 5 mm biopsy punch. The whole optic nerve was collected using clean dissection scissors. The fibrous sclera, optic nerve, and cornea samples were cut into smaller portions (about 1 mm^2) using a scalpel to aid in subsequent homogenization. After dissection all tissues were immediately frozen in liquid nitrogen for storage at −80°C until RNA extraction.

2.3. RNA extraction and whole genome expression processing

RNA was extracted from each tissue sample using the mirVanaTM total RNA extraction kit (Ambion, Austin, Texas, USA) following the manufacturer's protocol. The tissue samples were homogenized at 4°C in Ambion lysis buffer using a Bead Ruptor Tissue Homogenizer (Omni, Kennesaw, Georgia, USA) with 2.38 mm metal bead tubes using the following machine settings: 4 cycles × 30 s at speed 4.0 with 30 s break between cycles. Quality control for each sample included measuring RNA concentration and 260/280 nm ratios using a Nanodrop® (Invitrogen, Carlsbad, California, USA). The ocular tissue RNA samples were labeled and amplified using the Illumina® Total Prep kit (Ambion, Austin, Texas, USA). RNA samples were hybridized to Illumina® HumanHT-12 v4 Expression BeadChips (San Diego, California, USA), which target over 25,000 annotated genes (over 48,000 probes). All protocols were performed following the manufacturer's recommendations. Twelve samples per chip were processed and chips were run in two batches, each containing samples of at least two tissue types. The first batch ($N = 51$) included all adult and fetal retina/RPE, choroid and sclera samples. The second batch included all adult and fetal cornea and optic nerve samples ($N = 24$).

2.4. Whole genome expression analyses

The microarray data preprocessing procedures were conducted separately for each of the two batches (See Section 2.3). After the data was generated for each batch, background noise was subtracted from the intensity values using the Illumina® GenomeStudio program. The data was exported from GenomeStudio and log2 transformed. Sample outliers were determined by principle component analyses using the Hoteling's T2 test (Hotelling, 1931) (at 95% confidence interval) and removed from further analyses.