Retinal gene expression responses to aging are sexually divergent

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Purpose: Sex and age are critical factors in a variety of retinal diseases but have garnered little attention in preclinical models. The current lack of knowledge impairs informed decision making regarding inclusion and design of studies that incorporate both sexes and/or the effects of aging. The goal of this study was to examine normative mouse retina gene expression in both sexes and with advancing age.
Methods: Retinal gene expression in female and male C57BL/6JN mice at 3 months and 24 months of age were compared for sex differences and aging responses through whole transcriptome microarray analysis. Sex differences and age-related changes were examined in the context of cellular pathways and processes, regulatory patterns, and cellular origin, as well as for overlap with described changes in retinal disease models. Selected age and sex differences were confirmed with quantitative PCR.
Results: Age-related gene expression changes demonstrated commonalities and sexually divergent responses. Several cellular pathways and processes, especially inflammation-related, are affected and were over-represented in fibroblast, microglial, and ganglion cell–specific genes. Lifelong, and age-dependent, sex differences were observed and were over-represented in fibroblast-specific genes. Age and sex differences were also observed to be regulated in models of diabetic retinopathy, glaucoma, and other diseases.
Conclusions: These findings demonstrate that most age-related changes in retinal gene expression are sexually divergent and that there are significant sex differences in gene expression throughout the lifespan. These data serve as a resource for vision researchers seeking to include sex and age as factors in their preclinical studies.

Age and biologic sex are two important factors that influence retinal structure and function, as well as susceptibility to retinal diseases [1,2]. There is little published data regarding normative changes to the retina across the lifespan and no direct comparisons of the aging response in men and women. With the growing appreciation of the contribution of aging processes to disease development and progression, which has been termed geroscience [3], and the importance of sex as a biologic variable [4,5], there is an urgent need to compare and contrast retinal aging in both sexes. Currently, our understanding of the effects of age and sex on retinal function and their interactions with disease pathobiology to guide design of preclinical animal studies is lacking.

Normative sex differences in ocular anatomy, physiology, and visual performance may contribute to dissimilarities in retinal disease susceptibility [2,6,7]. Several clinical studies have reported that women have thinner retinas compared to men [8], while ganglion cell damage in pediatric multiple

sclerosis is more pronounced in young men compared to women [9]. Given that aging is the primary risk factor for common vision-threatening diseases, such as glaucoma and age-related macular degeneration (AMD) [10,11], and there is evidence for higher incidence and severity of late stage AMD in women [12-14] (although this is not always observed [15]), sex differences could also interact with age-related changes in disease processes. For example, female mice demonstrate more profound retinal damage with aging than male mice in experimental glaucoma models [16]. Intriguingly, this damage can be prevented in male mice by C1qa deletion but not in female mice. In rats, a larger electroretinography (ERG) response is evident in cycling versus reproductively senescent female rats, suggesting a beneficial effect of an intact estrus cycle on retinal function [17]. Additionally, a history of hormone replacement in women can also affect retinal disease prevalence, further supporting the potential role of sex hormones in age-related vision diseases [18,19].

Retinal sex differences and sexual divergences with aging most likely extend down to the molecular level. Efforts have also been begun to investigate the interactions of aging and sex on gene expression in the human central nervous

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system, including the brain and the eye [20,21], but there is little information on the primary preclinical model used in vision research, mice. The aim of this study was to examine sex-dependent differences and divergences with aging in the transcript profiles of the mouse retina. These data will serve as a resource for investigators incorporating both sexes in preclinical studies, especially of age-related retinal diseases. For the remainder of this report, the terminology follows recently proposed definitions [5] of sexual dimorphisms as binomial differences between male mice and female mice throughout life (e.g., the presence of a Y chromosome in male mice). Sex differences are different averages between male mice and female mice that exist throughout life (e.g., gene expression level of a particular gene). Sex divergences are differential responses to stimuli and events, such as aging, between the sexes. These categories are not exclusive but provide a structure and terminology to accurately categorize comparisons between male mice and female mice.

METHODS

Animals: The authors confirm adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research for all animal studies. All animal studies were approved by the Pennsylvania State University Animal Care and Use Committee and in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Male and female C57BL/6JN mice aged 3 (young), 12 (adult), and 24 (old) months were purchased from the National Institute on Aging colony at Charles River Laboratories (Wilmington, MA). Mice were housed in the Pennsylvania State University College of Medicine Hershey Center for Applied Research barrier facility in ventilated HEPA-filtered cages with access to standard rodent chow diet and water (Harlan 2918 diet, irradiated) ad libitum. In this facility, all animals are free of *Helicobacter* and parvovirus. After 1 week of acclimation, male mice were euthanized. In the female mice, estrous cycle staging was monitored by daily vaginal lavage, as previously described [22,23], for 3–4 weeks, and the female mice were euthanized during diestrus. Mice were euthanized by cervical dislocation and decapitation. CO_2 was not used to avoid differential effects between young and old. Retinas were rapidly dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until analysis.

RNA isolation: RNA preparation from retinas was performed according to standard methods [AllPrep DNA/RNA Mini (Qiagen, Germantown, MD)] as previously described [22,24]. RNA quality was assessed with the RNA 6000 Nano LabChip with an Agilent 2100 Expert Bioanalyzer (Agilent, Palo Alto, CA). Only samples with RNA integrity numbers greater than

7 were used in subsequent studies. RNA concentration was assessed with relative fluorescence using the RiboGreen assay (Invitrogen, Carlsbad, CA).

Microarray analysis: Transcriptomic analyses were performed on retina samples derived from young and old male and female mice (n=4/group, N=16) using Illumina Mouse Ref8 microarrays (Illumina, San Diego, CA) according to standard methods and as previously described [25,26]. Twelve-month-old adult mice were included at the confirmatory, quantitative PCR (qPCR), stage.

Arrays were quality control checked, and initial data analysis using average normalization with background subtraction was performed in GenomeStudio (Illumina). The full microarray data set has been deposited in the Gene Expression Omnibus, accession# GSE95220. Data analysis was performed in GeneSpring GX 14.8 (Agilent). Using detection p values generated by GenomeStudio, probes were filtered for only those with present calls and signal ≥50 in 100% of the samples in at least one of the four experimental groups (male/female, young/old). This ensured that transcripts not reliably detected in any group were excluded from statistical analysis, and that genes potentially expressed in only one experimental animal group were retained. A two-way ANOVA design was used to identify transcripts differentially expressed with the factors of age or sex and those with interactions of the two factors. Pairwise post-hoc analysis (Student–Newman–Keuls, p<0.05) was performed on the genes with a statistically significant effect (p<0.05) of age, sex, or interaction effect. A pair-wise absolute value fold-change cutoff of |1.2| was used in accordance with the standards for microarray analysis [27]. Two rounds of statistical thresholds and fold-change cutoffs were used to produce gene lists, with the lowest rate of type I and type II errors [28]. Venn diagrams and heat maps were generated with GeneSpring software. The full microarray data set is available through the Gene Expression Omnibus (GSE95220).

Bioinformatic analysis and visualization: Pathway, function, regulator, and cell-specificity analyses were performed using Ingenuity Pathway Analysis (IPA) software (Qiagen, Redwood City, CA) and database (March 2017 release). Bioinformatic analysis was conducted on all sets of pair-wise comparisons that passed statistical and fold-change cutoff thresholds. Complete gene lists for all pair-wise comparisons used are provided in Appendix 1 and Appendix 2. Retina cell–specific gene lists were manually derived from previous reports [29,30] (Appendix 3 and Appendix 4) and then imported into IPA for analysis of statistical over-representation. For the pathway, process, and regulatory analyses, an overlap p value and an activation *z*-score were computed [31].

For the cell-specificity analysis, only the p value was calculated as a *z*-score is not applicable. The p value was calculated using Fisher's exact test with Benjamini-Hochberg multiple testing correction based on overlap between genes in the list and known genes pertaining to a particular function, targets of a transcriptional regulator, or the imported gene list. The activational *z*-score is computed, if there is a significant over-representation, to infer likely activational states of a function or upstream regulator based on the direction of changes in the gene list and literature-derived functional or regulation directions [31]. A *z*-score cutoff of >|2| was applied to limit lists to only those functions and regulators with considerable activation (positive *z*-score) or inhibition (negative *z*-score) for the figures. A *z*-score approach identifies functional coordination in the patterns of gene expression that a p value for enrichment alone does not provide. For example, are the genes predicted to induce a function upregulated and those that inhibit this function downregulated? Comparisons to previously published gene lists from disease model studies were imported into the IPA software to concatenate different identification forms (gene symbols, GeneID numbers, and gene names). Overlapping genes were then determined and statistically assessed with an exact hypergeometric probability (Appendix 5).

Quantitative PCR: Confirmation of gene expression levels was performed with qPCR as described previously [24,25]. cDNA was synthesized from purified RNA (n = 7–8 for each age and sex group; male/female, young/adult/old) with the ABI High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA) from 500 ng RNA. qPCR was performed with gene-specific primer probe fluorogenic exonuclease assays (Appendix 6; TaqMan, Life Technologies, Watham, MA) and the QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems). Relative gene expression was calculated with ExpressionSuite v 1.0.3 software using the $2^{-\Delta\Delta Ct}$ analysis method with GAPDH as an endogenous control. Statistical analysis of the qPCR data was performed using SigmaPlot 12.5 (SyStat Software, San Jose, CA). Two-way ANOVA analyses were performed with the factors of sex and age. Post-hoc pairwise comparisons with factors of sex and age and interactions of both factors were performed with the Student–Newman–Keuls test with α<0.05. Benjamini-Hochberg multiple testing correction was applied to the F-test result to correct for the number of transcripts analyzed.

RESULTS

To identify age- and sex-related differences in retinal gene expression, young (3 months) and old (24 months) retina samples from male and female mice were compared with microarray analysis (n = 4/group). Young female mice were euthanized during diestrus, and old female mice were confirmed to be in reproductive senescence (permanent diestrus).

Microarray-based transcriptome analysis revealed age- and sex-related differences in retina gene expression: Of the 25,697 probes on the microarray, 10,251 passed filtering as expressed in all of the samples in at least one of the experimental groups (Figure 1A). To compare age- and sex-related differences in retinal gene expression, a two-way ANOVA with factors of sex and age was performed with the pair-wise Student–Newman–Keuls (SNK) post-hoc test. Genes found to be statistically significant by ANOVA and the SNK post-hoc test were further filtered for only those with a |>1.2| fold change in that pair-wise comparison. More genes were regulated with aging in male mice (539 genes) than in female mice (251 genes). Comparing the age-related changes within each sex (female old versus female young and male old versus male young) revealed 128 common genes regulated with aging in both sexes (intersection, Figure 1B). These sex-common age-regulated genes were regulated in the same manner (i.e., induced or reduced with aging in both female and male mice) for 125 of the 128 genes (98%, p<0.00001, chi-square). Of note was that the majority of the age-related changes were sex-specific (81%; Figure 1B). Examining sex differences within an age group (e.g., male young versus female young and male old versus female old) revealed the majority to be age specific (Figure 1C). Those sex differences common between young and old age comparisons were coordinately regulated 98% of the time (p<0.00001, chi-square). Examining the chromosomal location of those 125 genes with coordinate regulation, only nine were on sex chromosomes (eight X and one Y) with the remainder located on autosomes. Included in these sex differences are *Xist*, X-encoded and present only in female mice, and *Eif2s3y* and *Jarid1d*, Y-encoded and present only in male mice, which are more properly classified as sexually dimorphic. All differential age- and sex-related gene expression is presented in heat map form in Figure 1D, and full gene lists from each pairwise comparison are given in Appendix 1 and Appendix 2.

qPCR confirmation of differential gene expression: To confirm age- and sex-dependent changes in retinal gene expression, a selection of targets identified in the microarray was orthogonally validated using qPCR. An adult (12 months of age) group was added for these confirmations to

observe changes at mid-life. Genes selected for confirmation demonstrated age-related changes in both sexes that were more pronounced in one sex, gene expression changes with age that occur only one sex, or life-long sex differences/dimorphisms. For age-related changes that were more pronounced in one sex or the other, *Cx3cr1, Rdh9,* and *Edn2* (Figure 2A) were examined, and in all cases, statistically significant effects of age and sex (two-way ANOVA, p<0.05, Benjamini Hochberg Multiple Testing Correction [BHMTC]) were observed with pairwise post-hoc differences between the sexes only at old age for *Cx3cr1* and *Edn2*. For age-related changes observed in only one sex, *Ccl21b, Lcn2, Gbp4,* and *Rgr* were examined, and in all cases, a statistically significant interaction effect of sex and age was observed (two-way ANOVA, p<0.05, BHMTC; Figure 2B). *Ccl21b, Lcn2,* and *Gbp4* gene expression increased with aging only in female mice while *Rgr* increased with aging only in male mice. For sex differences throughout life, *Ddx3y, Rtn4,* and *Hes5* were

examined (Figure 2C). Sex effects were evident, along with some minor age-related changes, throughout life in all three genes. Although these qPCR confirmations are not exhaustive of all the microarray results, in all cases, they confirm the observations of the microarray analysis in larger sample numbers and with an intermediate age (12 months) group.

Pathway, processes, and regulators associated with age and sex differences: To identify patterns of gene expression changes with age and sex differences in the context of pathways and biologic processes, tertiary analysis was performed using the differentially expressed gene lists and the Ingenuity database of results from thousands of publications. Upregulation of immune response and inflammatory pathways, including Tec kinases, and the complement system (Figure 3A), were evident in the retinas of female mice and male mice with aging, while induction of the epidermal growth factor (EGF) and oncostatin M signaling pathways was observed only in female mice with aging. The only pathway with
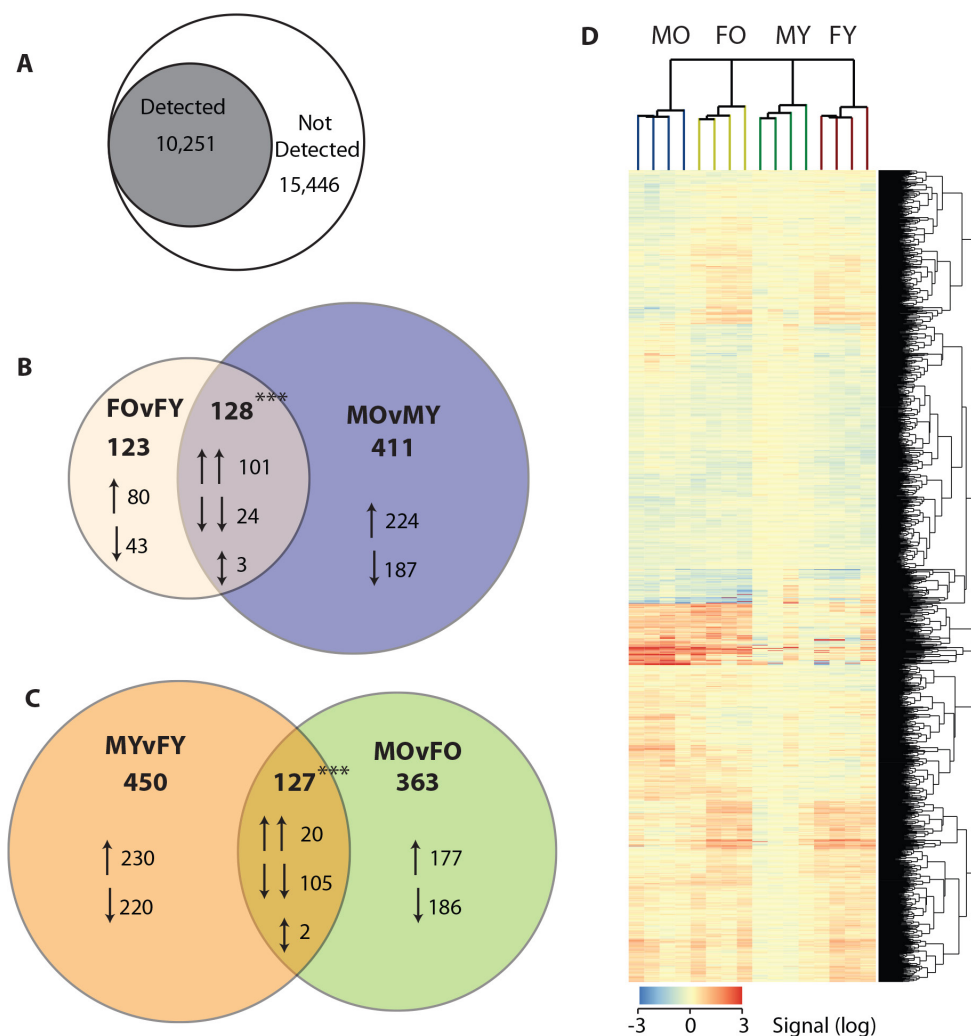

Figure 1. Differential sex- and age-related retina gene expression in the microarray analyses. **A**: Of the 25,697 probes on the microarray, 10,251 were positive for corresponding gene expression in the retina, i.e., passed all filtering. **B**: Comparison of pair-wise age-related changes in both sexes with the number of genes, direction of change, and intersections in respective groups (FO = female old, FY = female young, MO = male old, MY = male young). **C**: Sex differences in gene expression in young and old mice. **D**: Hierarchical clustering of all gene expression difference with age or between sexes. Samples segregated by sex and age.
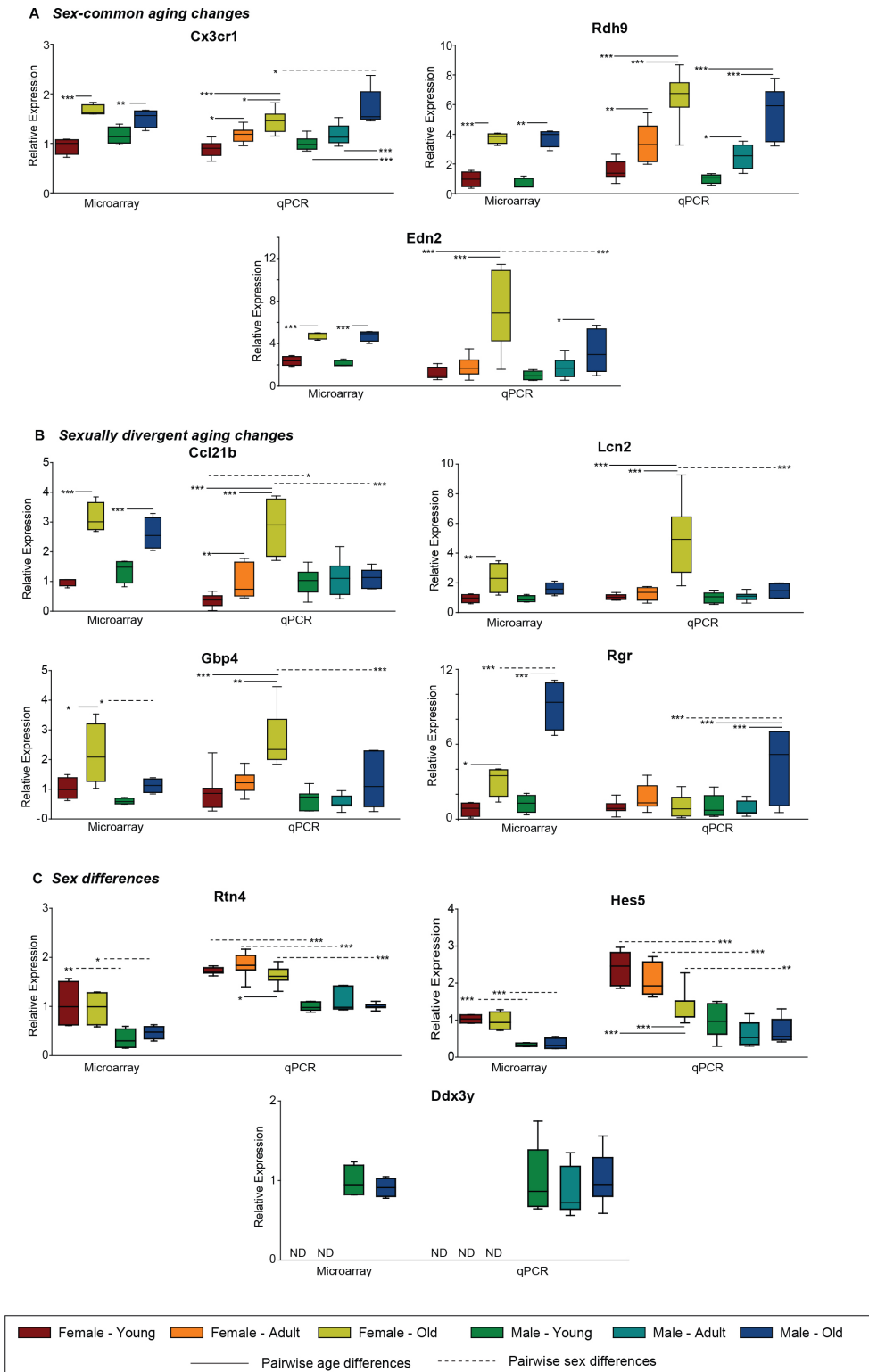
Figure 2. Confirmation of differential sex- and age-related retina gene expression with qPCR. Quantitative PCR (qPCR) analysis of selected targets identified in the microarray study confirmed differentially expressed retina genes that are associated with aging in female and male animals (**A**), sexually dimorphic aging changes (**B**), and changes related to sex differences (**C**). ND = not detectable. Data are shown as fold changes relative to young male animals for the microarray and qPCR data. Box plots denote median values with 25–75th percentiles quartiles, and whiskers define the 10–90th percentiles; n = 7–8 samples per group, for a total number of 47 samples analyzed. P values were determined with two-way ANOVA (age × sex), followed by the Student–Newman–Keuls post-hoc test. *p<0.05, **p<0.01, ***p<0.001. Solid lines denote comparisons of age-related changes within a sex group, and dashed lines are comparisons of sex-related differences within an age group. Cx3cr1 = chemokine (C-X3-C motif) receptor 1; Rdh9 = retinol dehydrogenase 9; Edn2 = endothelin 2; Ccl21b = chemokine (C-C motif) ligand 21B; Lcn2 = lipocalin 2; Gbp4 = guanylate binding protein 4; Rgr = retinal G protein coupled receptor; Ddx3y = DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked; Rtn4 = reticulon 4; Hes5 = hairy and enhancer of split 5.
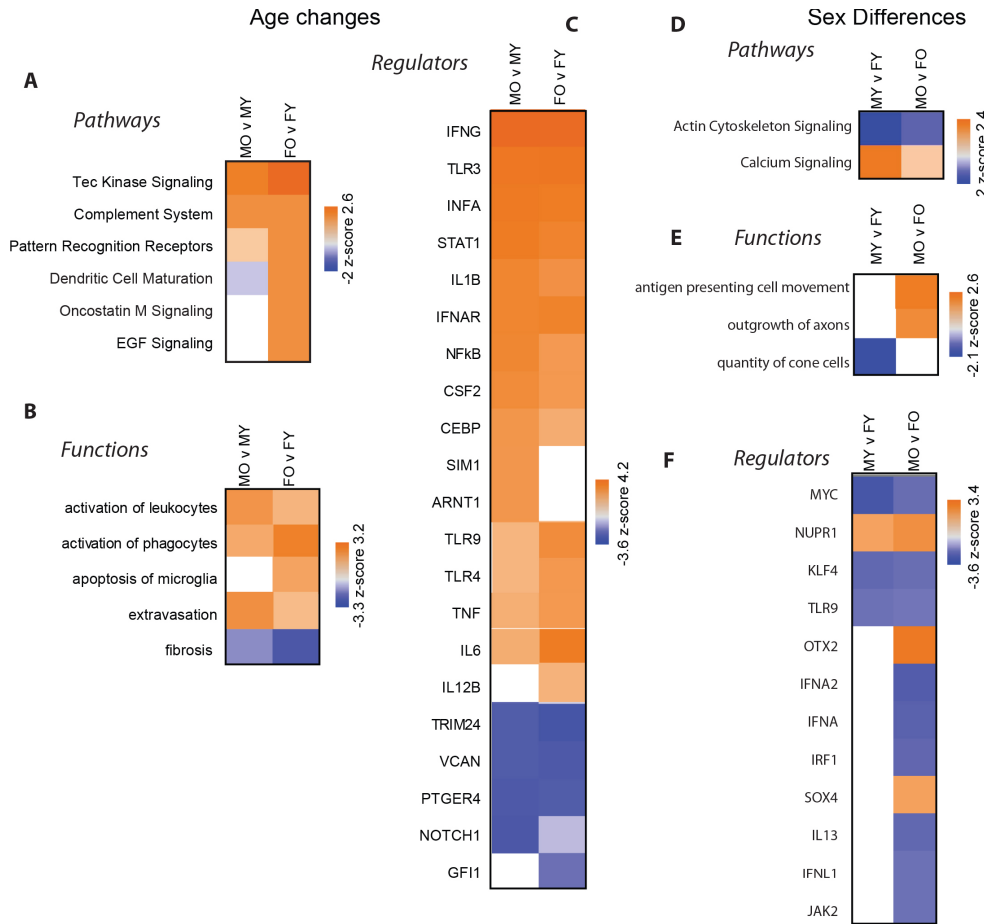
Figure 3. Pathway, function, and regulatory analysis of sex- and age-related transcriptomic changes. Age- (**A**) and sex- (**B**) related changes in retinal gene expression were analyzed with the Ingenuity Knowledge Base for differentially regulated pathways, functions, and regulators. Significantly over-represented functional or pathway processes (right-tailed Fisher's exact test, p<0.05) are shown in the heat map with coloring according to the computed *z*-score. A regulation *z*-score was calculated based on prior knowledge of known regulatory functions and direction of changes in the current data set, and a *z*-score >2 or a *z*-score <2 indicates significant activation or inhibition of a pathway or process, respectively. Note that increased immune and inflammatory responses with aging are evident in female and male animals. Selected pathways, regulators, and functions are presented. Full lists are included in Appendix 7 and Appendix 8.

statistically significant and opposing activation between the sexes was dendritic cell maturation, which was suppressed in male mice and induced in female mice. For functional groupings, age-related induction of inflammatory processes and suppression of fibrosis were observed in male mice and in female mice (Figure 3B). Gene regulator analysis revealed activation of a large number of upstream regulators related to the immune and inflammatory responses with aging in both sexes but with some findings specific to one sex (Figure 3C). Several transcriptional regulators were inhibited in both groups. Only a selection of pathways, functions, and regulators are presented. Full listings are in Appendix 7.

Sex differences in gene expression were also analyzed for over-representation of pathways, functions, and potential upstream and downstream regulators. Deactivation of actin cytoskeleton signaling and greater activation of calcium signaling in male mice compared to female mice was evident at young and old ages (Figure 3D). Gene expression patterns indicative of greater antigen presenting cells, and axon outgrowth were observed in male mice compared to female mice while genes indicative of cone cell number were

suppressed in male mice at the young age time point (Figure 3E). Gene expression regulators demonstrated a more diverse pattern of responses with sex differences common across ages and specific to one age or the other (Figure 3F). Full lists of pathways, functions, and upstream regulators are in Appendix 8.

Cellular origins of sex and age differences: Two recent studies reported retina cell type–specific transcriptomes from RNA sequencing of single cells or isolated cell types [29,30] (Appendix 3 and Appendix 4). We extracted the gene sets from these reports to generate lists of cell type–specific genes for 12 retinal cells (rods, cones, Müller cells, pericytes, microglia, amacrine cells, astrocytes, bipolar cells, endothelium, fibroblasts, ganglion cells, and horizontal cells). Using these lists as references, we sought to determine whether there are more age- or sex-related differences associated with specific cell types than would be expected by random chance with the goal of localizing some of the gene expression differences to specific cell types and determining whether specific cell types were the predominant site of age or sex differences. Comparing a single cell type with gene sets of the age-related

changes revealed a high level of enrichment in fibroblast- and microglial-specific genes, and to lesser extent retinal neurons, such as ganglion, cone, rod, and amacrine cells (Figure 4A). Examining the over-representation of cell-type-specific genes associated with sex differences showed enrichment in genes expressed only by fibroblasts and in several retinal neuron types (Figure 4B).

To provide context for the potential importance of considering sex and age effects on retinal gene expression, gene sets from previous reports that used mice as a retinal disease model were compared to the sex and age differences described here. Selected studies that investigated aging [32,33], diabetic retinopathy [34-36], axonal injury [37], or glaucoma [38,39], or a database of genes with known relevance to retinal diseases [40] were examined. To determine whether there was more overlap with genes identified as differentially expressed in these studies, an exact hypergeometric test was performed for the overlap between these studies and the age- or sex-related changes observed in the present study. Significant overlap with sex- or age-related differences was observed with all of the studies except those that examined aging [32,33] (Figure 4C).

DISCUSSION

Given the importance of sex [4,5] and age [3] as biologic variables in health and disease, preclinical retinal research requires including these variables to adequately address the human condition. For example, age-related disease rates such as those for glaucoma [41], AMD [42], and microvascular complications from type I diabetes [43] may be higher in women. In addition, illustrative of this potential interaction effect of aging and sex differences is diabetic retinopathy that may be more pronounced in men with advancing age [7]. The finding that a history of hormone replacement in women can also affect retinal disease prevalence suggests an impact of sex hormones on age-related diseases of the eye [18,19].

Previously, we demonstrated commonalities between age-related changes in the rat retina and those in diabetic retinopathy models [24]. In the current study, the retinal transcriptome was analyzed in young (3 months) and aged (24 months) male and female mice to explore the sex differences in retina gene expression with aging. With the renewed focus on understanding the role of sex differences and sexual dimorphisms in health and disease [4], these findings of normative commonalities and differences in the retinal response to aging in the most commonly used mouse strain can help guide study design and interpretation.

The findings presented here establish several points regarding retinal aging in male and female mice: 1) Although commonalities in the retinal aging response between the sexes exist, male mice and female mice are predominantly sexually divergent with aging, 2) life-long retinal sex differences are evident, 3) aging responses and sex differences originate from a variety of cell types and affect a range of cellular processes, and 4) age-related changes and sex differences in gene expression overlap with those observed in disease models. These findings have several implications for the design and performance of preclinical retinal research: 1) When using male and female mice, either in combined groups or as separate factors, study designs should plan for the significant sex differences in gene expression observed throughout life; 2) for age-related retinal diseases, consideration should be given to using aged mice or a range of ages as extensive changes in normative gene expression are occurring with age that may exacerbate or ameliorate disease phenotypes; and 3) these findings serve as a resource for investigators to determine whether genes, pathways, or processes of interest are regulated with age or between the sexes.

Although it was neither the goal nor the design of this study to mechanistically determine the origins of sex differences and divergences, a likely mechanism, among many, is the effect of sex hormones on the retina. Although we did not observe any expression of classical estrogen receptors (neither ERα nor ERβ), expression of the g-protein coupled estrogen receptor (GPER/GPR30) mRNA was detected. GPER is a membrane-bound receptor that acts independently of ERα and ERβ in response to estrogen binding to induce rapid signal transduction [44]. Our finding aligned with recent human retina RNA sequencing data that showed low or no expression of the ERs but the presence of GPER/GPR30 [45]. Previous immunohistochemistry studies suggest that ERs may or may not be expressed in the retina depending on the sex or age [46]. Given that estrogens in the retina can also signal through non-estrogen receptors [47,48], and the RPE expresses ERs [49], there are a variety of potential mechanisms by which estrogens could modulate retinal gene expression, which warrant further investigation. Combined with the presence of androgen receptors in the retina [50], sex hormones, and their changes with age, could directly impact gene regulation in the retina, giving rise to the sex differences and divergences observed here.

These findings also point to the need for additional studies of retinal sex differences and changes with aging. This study examined only one strain of mice, and examinations of additional strains, as well as common rat models, are warranted. The limited overlap with previous retinal aging studies [32,33] cannot be ascribed to strain differences as the past and present studies used the same mouse strain. All of
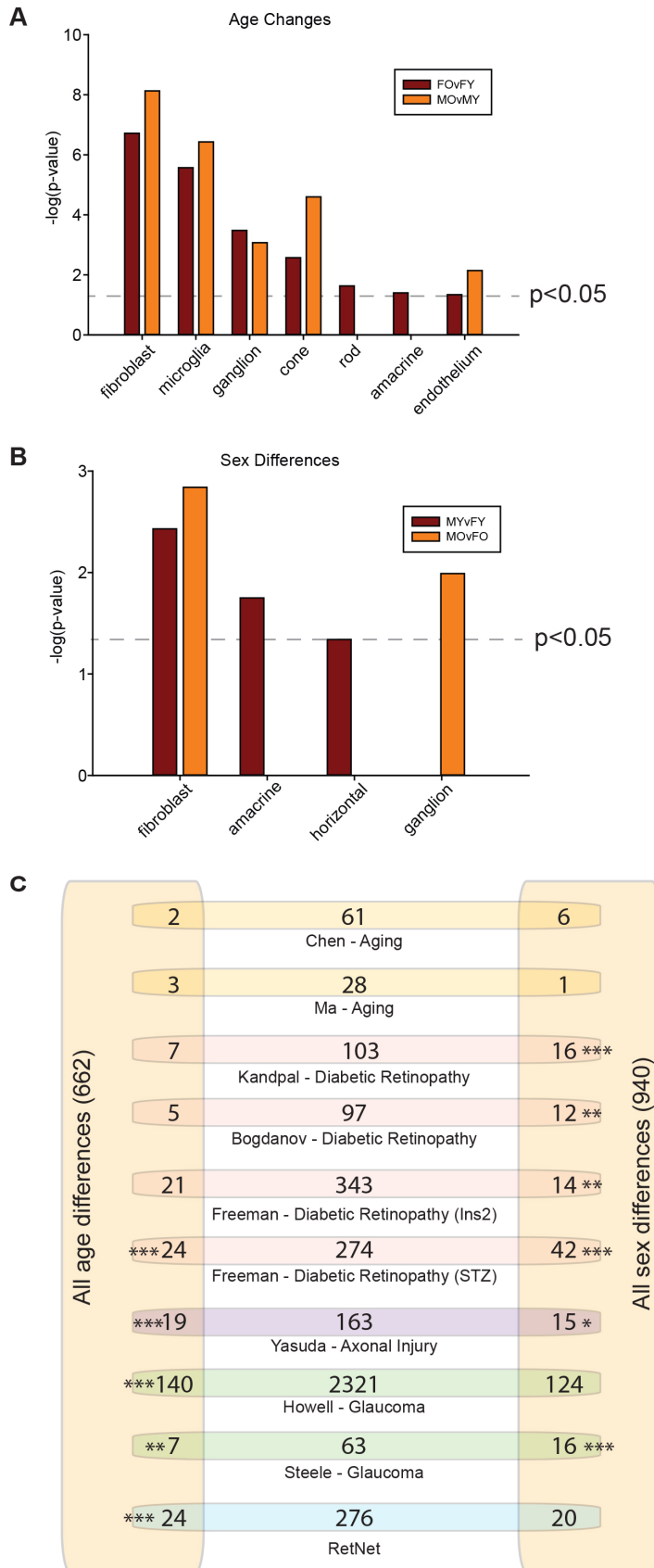
Figure 4. Enrichment of sex- and age-related changes in cell type–specific transcripts of the mouse retina. Retinal cell type–specific transcripts from previous reports [29,30] were compared to each pair-wise set of age- (**A**) and sex- (**B**) related changes, and p values were calculated using Fisher's exact test. Dashed lines indicate significant over-representations of specific retina cell types with sex- and age-dependent changes in gene expression. **C**: Comparisons to disease model differential gene expression data sets demonstrate significant overlap with the age- and sex- differences observed in this study (exact hypergeometric test, Benjamini-Hochberg multiple testing correction, *p<0.05, **p<0.01, ***p<0.001).

these studies used different microarray formats [two-color [32], short oligonucleotides (Affymetrix) [33], and single color long oligonucleotides (Illumina, present study)] which may be responsible for the limited common genes and precludes data analysis by the same bioinformatic methods. However, all three studies demonstrated induction of complement 3 (C3), with other, non-overlapping elements of the complement cascade induced in all three reports. Given that complement induction is observed with aging [51] and in a sexually divergent manner [52] in the brain, examination of the cellular source and function impact of retinal complement activation with aging is warranted. A more complete understanding of age-related changes in both sexes would also be gained by examining a number of ages across the lifespan. Additionally, analysis of isolated cell populations or single cells would provide greater insight into cell-specific differences.

In conclusion, this study demonstrates retinal age- and sex gene expression differences in mice. Moreover, the aging response is predominantly sexually divergent. These findings provide a new view on the degree and importance of considering the factors of age and sex in vision research and in studies of age-related retinal diseases in particular. These data also serve as a data resource for investigators to use in comparisons to data from their mouse model studies.

APPENDIX 1. AGING CHANGES.

To access the data, click or select the words "Appendix 1"

APPENDIX 2. SEX DIFFERENCES.

To access the data, click or select the words "Appendix 2"

APPENDIX 3. SIEGART GENE LISTS.

To access the data, click or select the words "Appendix 3"

APPENDIX 4. MACOSCKO GENE LISTS

To access the data, click or select the words "Appendix 4"

APPENDIX 5. DISEASE GENE LISTS

To access the data, click or select the words "Appendix 5"

APPENDIX 6. QPCR PRIMERS

To access the data, click or select the words "Appendix 6"

APPENDIX 7. SEX DIFFERENCES – PATHWAYS, REGULATORS, FUNCTIONS

To access the data, click or select the words "Appendix 7"

APPENDIX 8. AGE CHANGES – PATHWAYS, REGULATORS, FUNCTIONS

To access the data, click or select the words "Appendix 8"

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