Original Article

Differential Expression of Genes in the Cornea Compared to the Optic Nerve

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Abstract

Background: Identification of tissue-specific biomarkers can help us in finding the causes of disease. In this context, finding genes that are expressed in different tissues gives us guidance in cell differentiation.

Material and Methods: we examined genes with different expressions in both optic nerve and corneal tissues. First, we found differentially expressed genes on a dataset including 4 corneal and 4 optic nerve samples, then evaluated them on another independent dataset to validate the findings. Finally, we performed Enrichment Analysis and Network Evaluation of the obtained DEGs.

Results: we gained 6 genes that are highly expressed in the optic nerve than the cornea and 99 genes with higher expression in the cornea than in the optic nerve.

Conclusion: We saw traces of DEGs in the mechanism of action of the corresponding cells. In this study, we did not use the FDR method and thus we do not have false positive in final DEGs.

Keywords: Biomarker Discovery; Cornea; Optic Nerve; Differential Gene Expression Analysis.

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Introduction

The main goal of all body tissues is to maintain survival, but each tissue also has a different purpose and responsibility. By controlling the gene expression network in tissues, the amount of proteins in each tissue will be different from the other tissue, and as a result, tissues will have different functions [1]. Finding specific regulatory genes in a tissue helps us understand tissue function. By increasing our understanding and knowledge about the specific function of a tissue, we can take a step closer to finding the cause of specific diseases of that tissue. Also in creating artificial tissue, we need to control the regulatory network of gene expression by increasing knowledge about genes that are specifically expressed in tissues.

Until now, more than 20 corneal diseases are known. For instance, in Keratoconus, the cornea becomes thin and protrudes [2] with an incidence of approximately 1 per 2,000 in the general population [3]. However, Ma-dot-fingerprint dystrophy is defined as Map-dot-fingerprint basement-membrane abnormalities of the cornea that are common in the general population, affecting as many as 76 % of persons over age 50 and 42 % of persons of all ages [4]. Another case is Fuch's dystrophy (Fuchs endothelial corneal dystrophy), documented as FECD, which is characterized by progressive loss of corneal endothelial cells, thickening of Descemet's membrane, and deposition of extracellular matrix in the form of guttae. When the number of endothelial cells becomes critically low, the cornea swells and causes loss of vision. The clinical course of FECD usually spans 10–20 years [5].

By now, the main reason for these diseases is unknown. To understand their mechanism, it is essential to study their foundation in terms of molecular biology in the levels from genetic, epigenetic, transcriptomic, and proteomic. If there are specific genes expressed in a tissue, the diseases associated with that tissue will most likely be due to changes in those specific genes. The same idea applies to optic nerve disease [6].

Despite receiving medication and surgery, still some diseases of the cornea do not treat. This is the reason for the need for the artificial cornea. Having an artificial cornea for transplantation to patients whose treatment does not completely restore vision can be a good way to restore vision. Making an artificial cornea requires our knowledge of specific corneal genes to achieve a flawless cornea by controlling gene expression. In the research [7], gene expression data were provided for cornea, lens, iris, ciliary body, retina, and optic nerve. They compare genes in one tissue with other tissues and gain the signature of tissue, do this for all tissue. However, these genes have not been validated on other independent data. In addition, by comparing only two tissue, more detailed information could be achieved. The above encourages us to look for specific genes in different eye tissues. The aim of this article is to find specific genes of the two tissues of the cornea and optic nerve so that we can get a hole for making artificial tissue and to find the causes of diseases of these two tissues. For more detailed studies on finding specific genes for each tissue, that tissue should be compared with several other tissues. Here we are just one-step closer to the main goal by comparing the two tissues on the two datasets.

Material and methods

Herein, we designed a pipeline with the following steps. First, in order to find out genes that undergo a distinct transcription in each of both tissues, Differentially Expressed

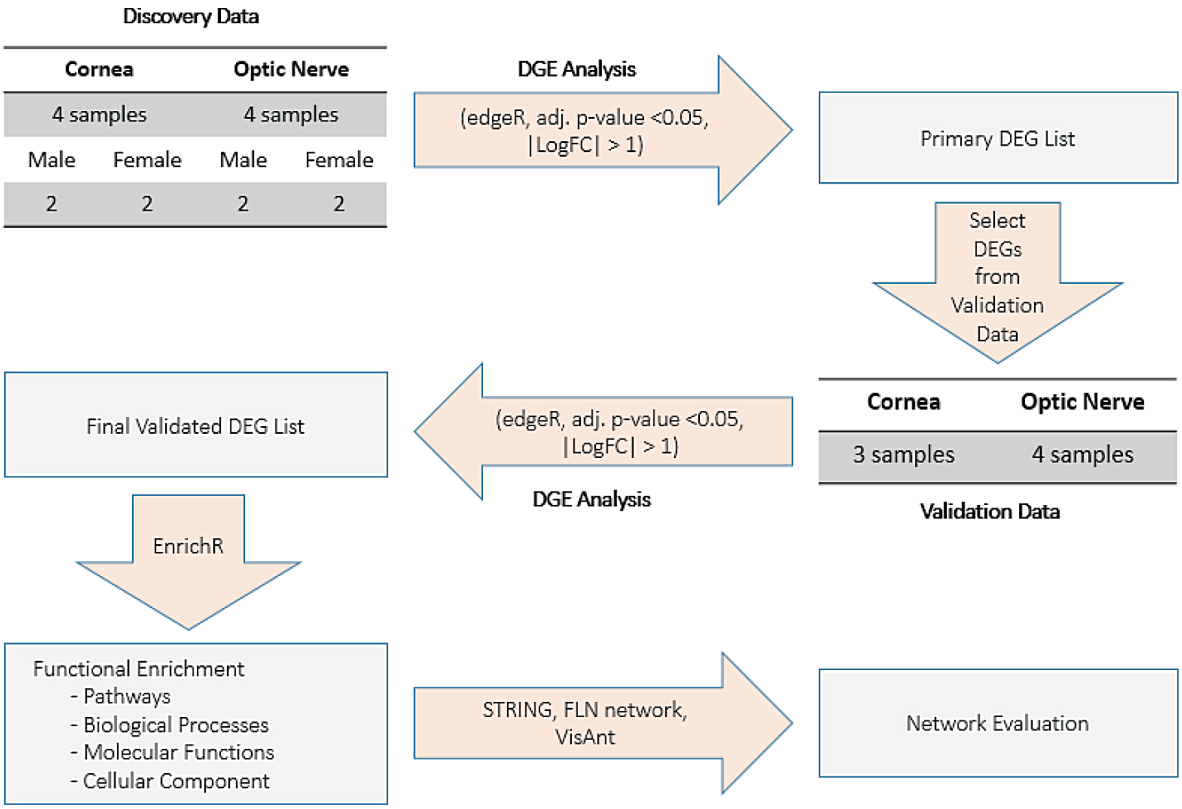


Figure 1: Research workflow. First, we find primary DEGs on discovery data, then select pre-specified primary DEGs from validation data. Next, to examine the functionality of candidate genes, we do enrichment analysis. Finally, the extracted modules are observed from a network topology viewpoint

Genes (DEGs) are specified. To extract DEGs without affecting the ethnicity and gender of the test subjects and to reduce the effect of the technique used for the test, we first perform the test on a dataset and examine the obtained primary DEGs on independent data set. Next, we evaluate the final DEGs using enrichment and network tools. This workflow is given in Figure 1.

Cohorts

We use two independent datasets in this project: Discovery and Validation. In the Discovery data, samples of both cornea and optic nerve tissues were taken from [7] with accession number GSE3023. The collector of this data used DNA Microarrays representing 30,000 human genes to analyze gene expression in some sub-tissues of the eye. Samples were harvested from autopsy donors within 24 hours of death. The age of the donors was between 30 and 85 years. Of the 4 corneal samples, 2 belong to men and two to women. In addition, the same ratio is established in 4 samples of the optic nerve (2 males, 2 females). For the confirmation of output DEGs, the Validation dataset was employed. Here, RNAseq samples of both cornea and optic nerve tissues were obtained from GSE159359 [8] achieved by Illumina NextSeq 500 platform. First, we plot result of hierarchical clustering to find sample noise, but according to these plots for Discovery and Validation data, we do not have

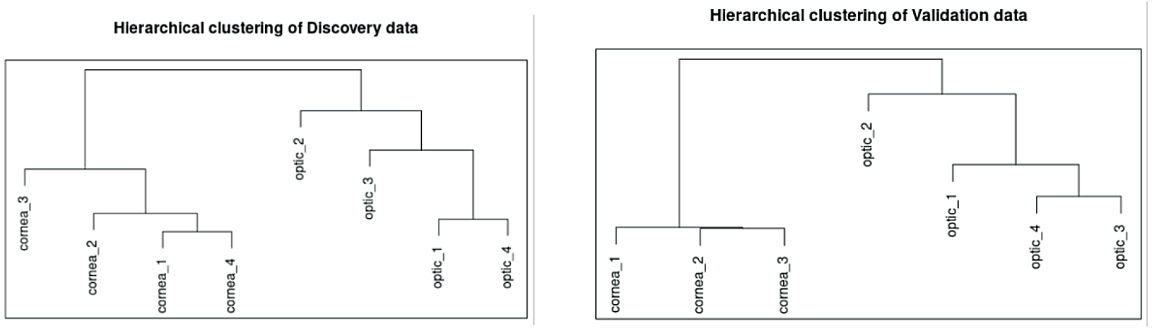


Figure 2: Hierarchical clustering of: Left: Discovery data. Right: Validation data

any sample noise (Figure 1).

In the data preparation phase to continue the process, we omit genes that had the amount NA for even one sample and genes with an average expression less than one in the validation data. We used TMM [9] method for normalizing Validated data.

Differential Gene Expression analysis

To perform Differential Gene Expression (DGE) analysis of discovery data, the edgeR [10] R package and its eBayes function were hired. At this step, we set the adjusted P value to less than 0.05 (adj. P value < 0.05, with Bonferroni method) and absolute Logarithm of Fold Change to be larger than one (|LogFC| > 1). This is the same for the DEG analysis of candidate genes in the evaluation stage.

Enrichment

To investigate the relationship between DEGs and the development of separate tissues, we performed enrichment analysis. We are interested in all significant pathways and biological processes that our output genes are involved in, and compare them with the activities of each tissue. This analysis was implemented using the EnrichR [11] tool, containing profiles of other complementary databases. Here, for the pathway associations, the KEGG [12], and for the gene ontology investigations (biological process, molecular function, and cellular component), the GO [13] was utilized. In all these inspections, we considered terms with a P value < 0.05.

Networks

As another complementary validation, we reconstruct PPI subnetworks of the up-regulated and down-regulated DEGs and investigate their physical interactions at the proteomics level from a network-based topological view. For this purpose, we utilized the human PPI network from the STRING [14] database and left all its parameters as default, that means Network type set to full STRING network, minimum required interaction score set to 0.4 and active interaction sources was considered a collection of Text mining, Experiment, Databases, Co-expression, Neighborhood, Gene Fusion and Co-occurrence. Moreover, in order to check the functionality of DEGs associated proteins, the Functional Linkage Network (FLN) [15] was used in which each node is a protein, and there is an edge between two nodes if there is evidence that nodes have a degree of the functional similarity. In this network, edges are weighted and predicted based on PPI interactions, gene expression profiles, literature mining, experimental techniques, and computational approaches.

Table 1: The number of samples for each tissue and for each data

Data	Accession number	Number Of Cornea Samples	Number Of Optic Nerve Samples
Discovery	GSE3023	4	4
Validation	GSE159359	3	4

Results

Statistics of Data

Due to the hierarchical clustering, we did not have any noise samples, and the statistics of data that used in the next steps, are provided in Table 1.

DEG analysis

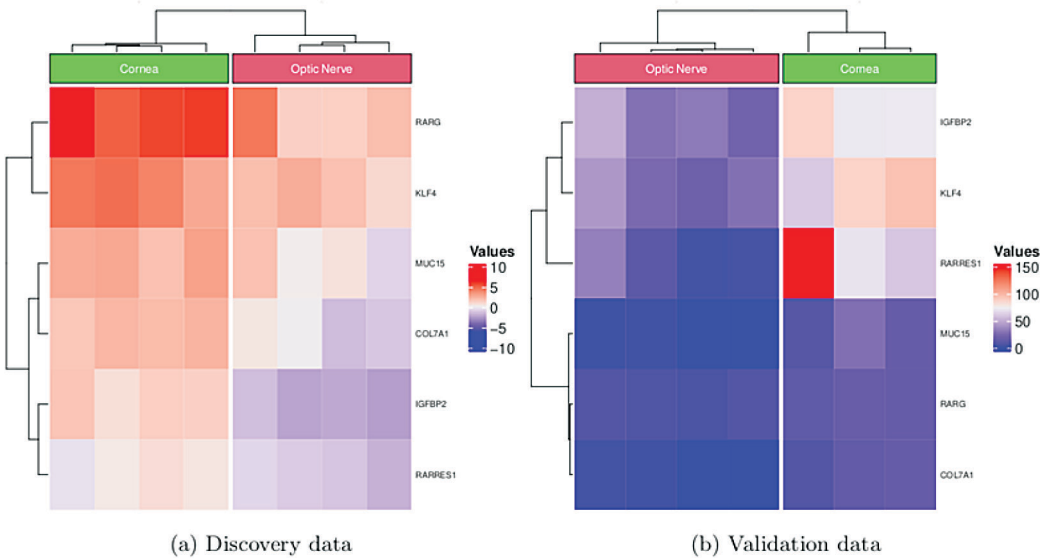
Performing differential gene expression analysis on the discovery data, resulting in a primary list in which 203 genes were identified as up-regulated and 391 as down-regulated genes. As previously described, afterward, we performed another differential expression analysis for these genes on the validation data. Finally, we obtained 6 down- and 99 up-regulated genes (Table 2). Heatmaps for the two trials (discovery and validation) are displayed in figures 3 (for down-regulated) and 4 (for up-regulated genes).

Assessment

Previously, we have found genes that have different levels of expression in the optic nerve and the cornea. Down-regulated genes are more expressed in the optic nerve than the other cells. In other words, they are less expressed in the cornea than in the other cells. Here, we examined the top pathways

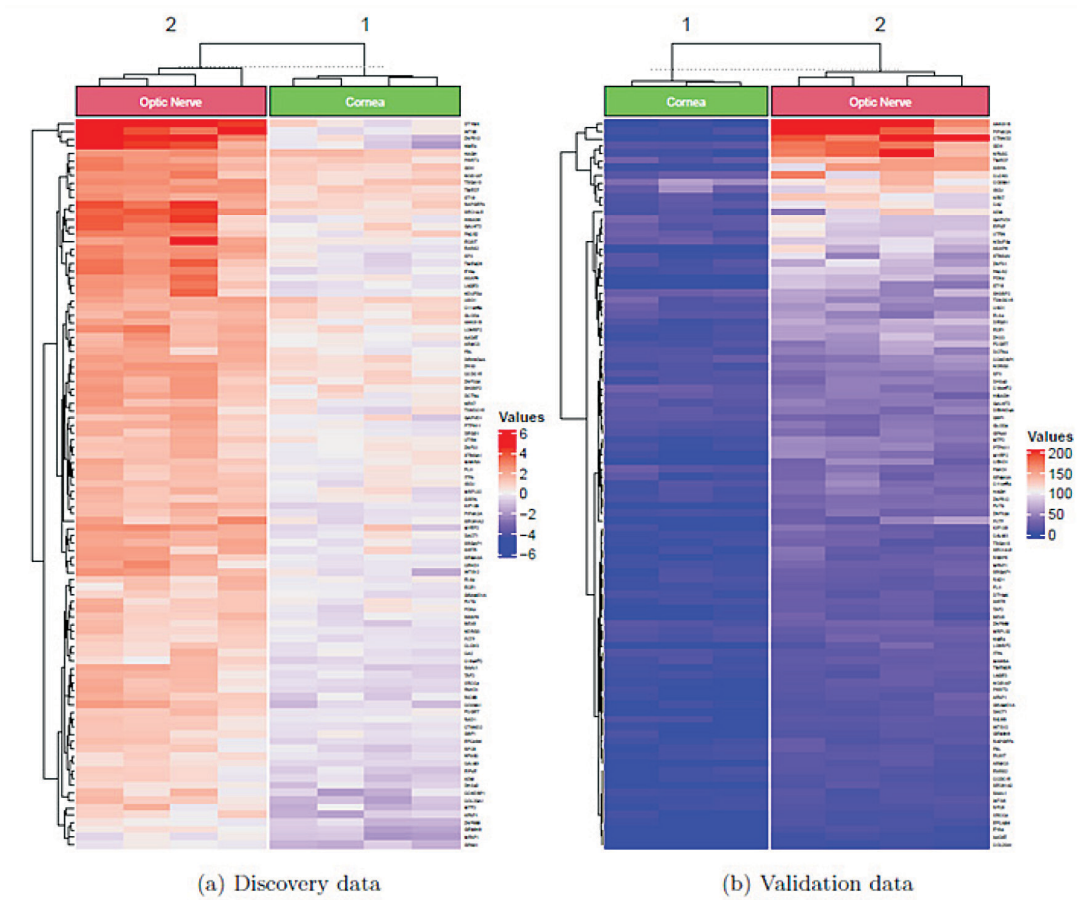
Table 2: Final DEGs. These DEGs were first obtained from the discovery data and verified with the validation data

genes that are more expressed in the optic nerve than the cornea	RARG, IGFBP2, KLF4, COL7A1, MUC15, RARRES1
genes that are more expressed in the cornea than the optic nerve	FUT9, ECE1, PACS2, TMED7, MTF2, NDUFS4, GDI1, NDRG3, ZNF512, SP3, DEGS1, GLOD4, CA2, FLI1, PDK4, MFAP1 ,NFASC, PRRT3, PTPN11, GALNT2, INTS12, EIF4E, ZHX3, NEK7, SIRPA, HAGH, MYEF2, DCTN4, GPAA1, DHX40, UTRN, SIRT5, NOS1AP, DENND4A, TAF2, ZNFX1, ZNF33A, GBF1, MANBA, GAPVD1, SRGAP1, ECSIT, ANKS1B, C11orf54, USO1, AKAP6, PIP4K2A, ELK4, MRPL32, COX6A1, ISCU, CLCN3, RFC5, FCGRT, SH3BP2, CTNND2, TMEM25, RAD1, ST18, EFCAB6, C16orf72, CCNDBP1, INTS5, ARMC3, TXNDC15, ZNF688, HIBADH, CALM3, RIC8B, KIF13B, ST8SIA1, ITPA, SEC61A2, CEND1, GRAMD1A, AADAT, DACT1, EARS2, NME4, CCDC15, COL20A1, FAHD1, SEMA3A, SEC14L5, RBBP5, GEMIN8, ADM, FBL, LAGE3, DTYMK, XRCC4, AFAP1, EYA4, RAPGEF4, TSGA10, PLTP, BEX5, SAAL1, LONRF2



(a) Discovery data (b) Validation data

Figure 3: Heat-maps for the final down-regulated (cornea-specific) DEGs in discovery (a) and validation (b) datasets



(a) Discovery data (b) Validation data

Figure 4: Heat-maps for the final up-regulated (optic nerve-specific) DEGs in discovery (a) and validation (b) datasets

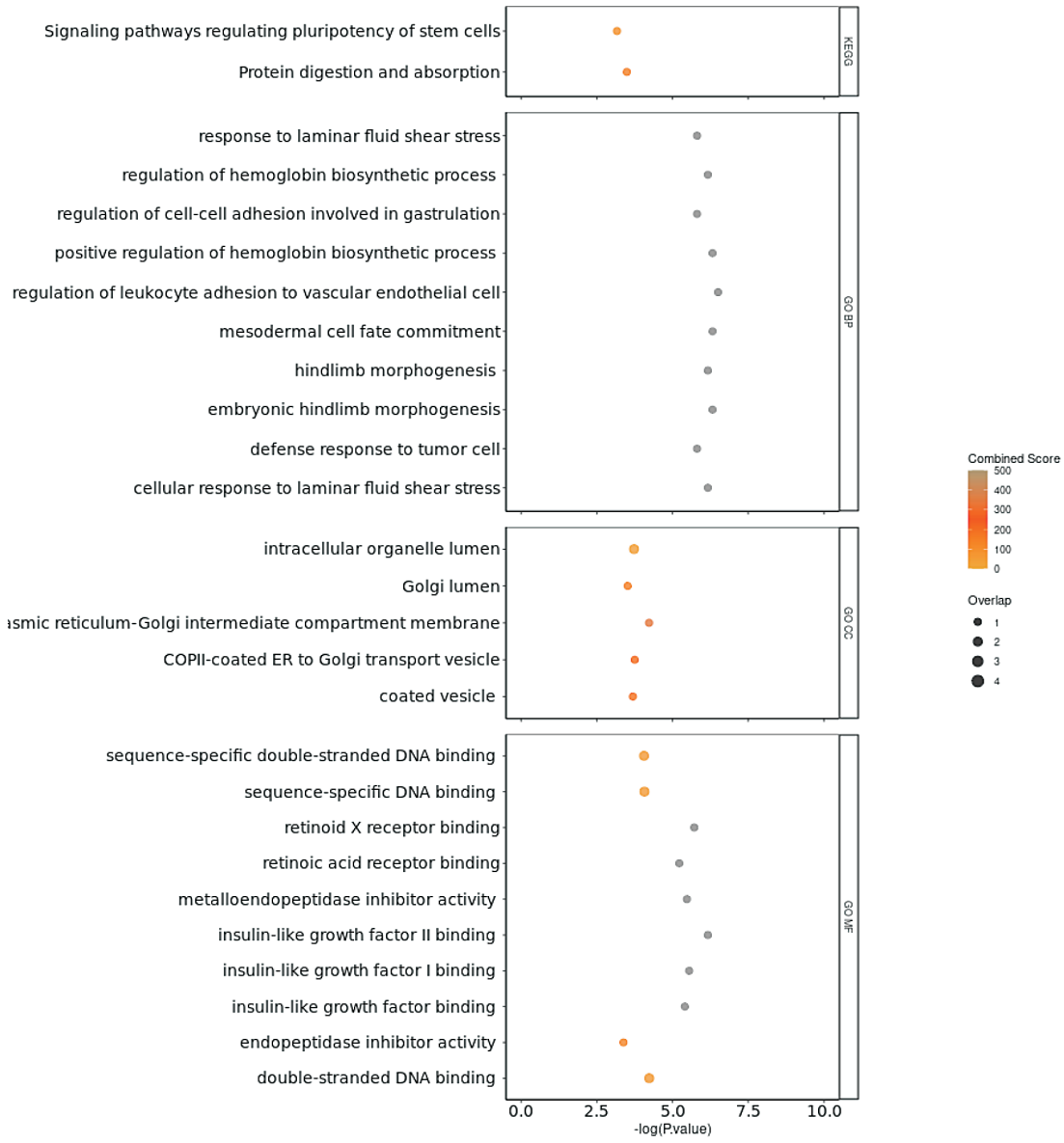


Figure 5: Enriched terms (pathway, biological process, molecular function, and cellular component) for the down-regulated genes

in both optic nerve tissue and the cornea (Figure 5). Moreover, the results for pathway, biological process, molecular function, and cell component enrichments for down- and up-regulated genes are presented in figures 5 and 6 respectively.

PPI and FLN networks

As detailed in the Materials and Methods section, for the genes with a different expression pattern between two tissues, we generated tissue-specific PPI and FLN sub-network for two purposes: First, to observe if they have physical interactions in making complex proteins acting in the tissue. Second, to examine if they have functional similarity which makes them alternatives in the action. The sub-networks were explored topologically to see their connectivity and density. This means that how much a sub-network is dense shows their tight connections and high

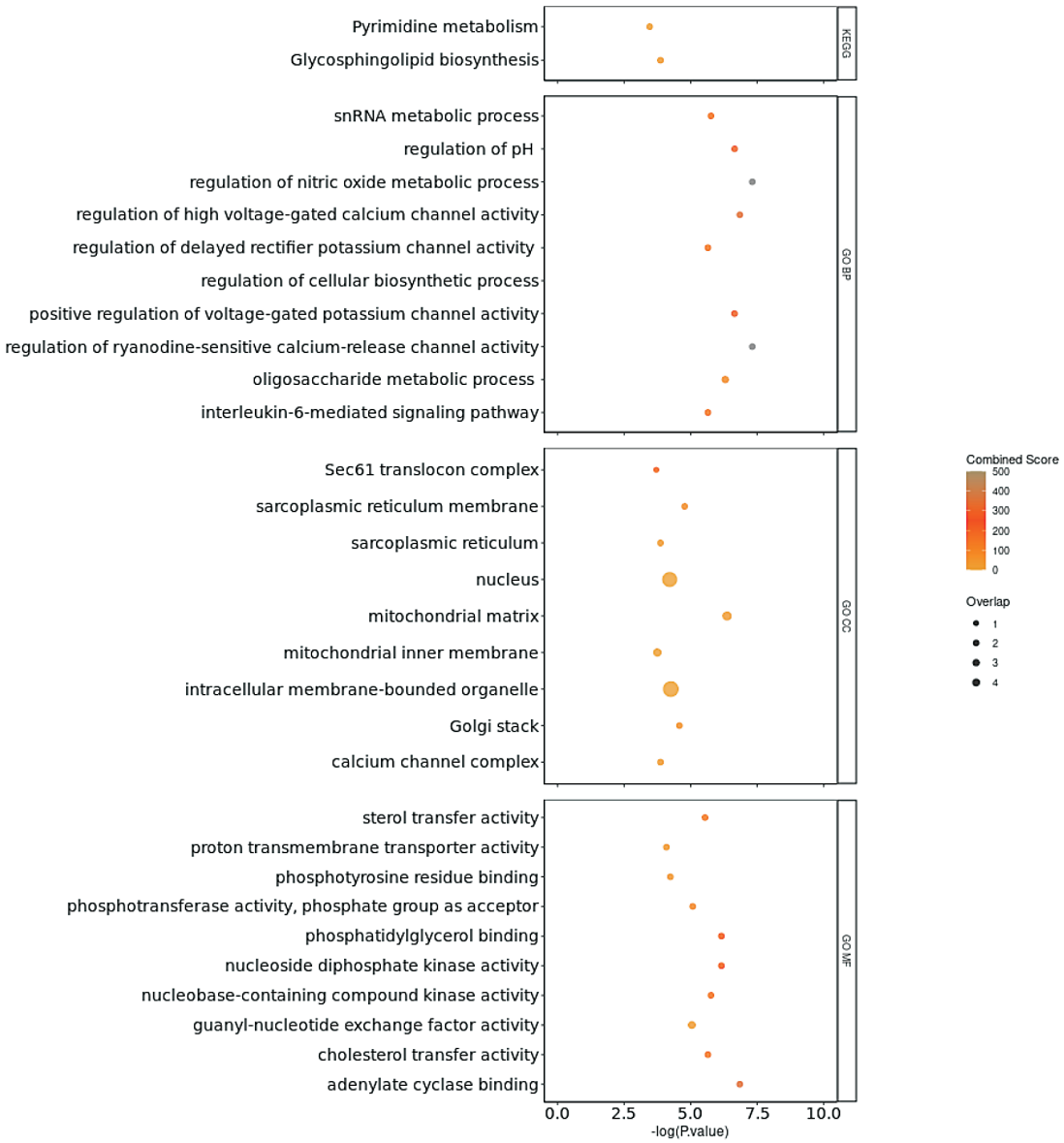
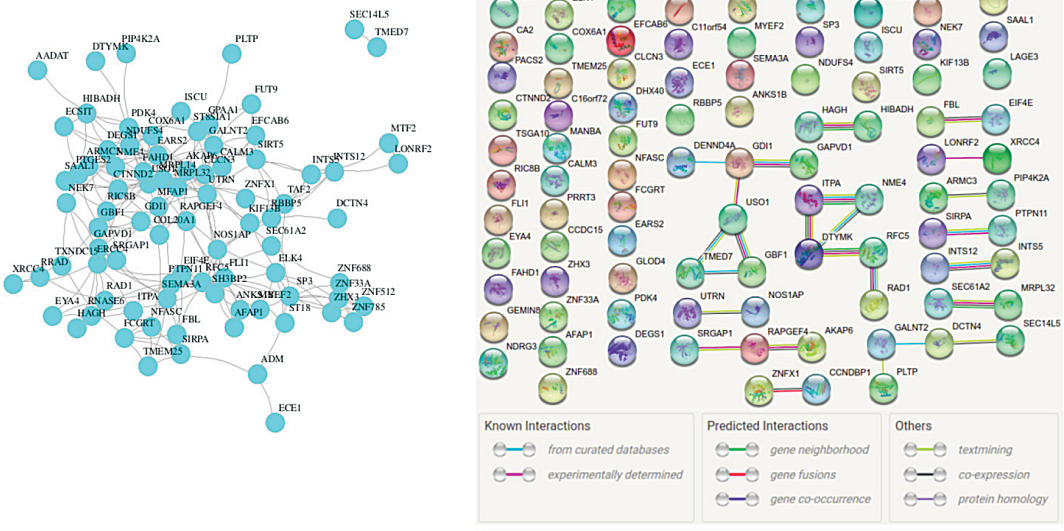


Figure 6: Enriched terms (pathway, biological process, molecular function, and cellular component) for the up-regulated genes

degree interactions that lead to a function and goal. It was seen that FLN sub-network of up-regulated genes contains 86 nodes and 244 edges, with shortest path length of 4 and density equal to 0.133. This exhibits that the extracted module contains components that are similar in the function. In the same way, the PPI subnetworks were surveyed.

Discussion

In the network assessment of down- and up-regulated genes, the results show the sparsity of the two tissues' PPI networks. This means that most translated proteins of DEGs do not interact with each other, which confirms our findings in the FLN subnetworks. When two proteins have similar conformation, it is imaginable that they do not have complementary biding sites to make them create complexes. Another assumption is that this is because of interactome incompleteness (unknown protein-protein interactions).[16] As the results of enrichment analysis show



(a) similarity network between up-regulated DEGs obtained from visANT

(b) network of up-regulated DEGs obtained from STRING

Figure 7: Networks of up-regulated DEGs

in Figure. 6, some of the up-regulated genes are involved in the regulation of stem cells pluripotency signaling pathways. As this pathway's name implies it is expected to be active in stem cells. Since Corneal Epithelial Stem Cells are located in the basal layer of the peripheral cornea, it is not also far from expectation to see up-regulation of genes participate in such pathways in the cornea compared to the optic nerve. In addition, the cellular component graph shows most of the up-regulated genes are located in Golgi and relevant cellular compartments which verifies up-regulation of protein synthesis. The stromal part of the cornea is comprised of the pseudo crystalline lattice of collagen fibers, proteoglycans, and the stromal cells that are also known as keratocytes. Keratocytes can differentiate repair fibroblasts and further differentiation can occur in repair fibroblast leading to myofibroblasts generation. Repair fibroblasts are capable of proliferation and active production of extracellular matrix. On the other hand, myofibroblasts are actively express different proteins [17, 18]. In this case, active protein synthesis and digestion are expected to be observed in the cornea.

Moreover, the human cornea is made up of at least six glycoproteins and glycosaminoglycans, and various glycoproteins. All these molecules together can build up a highly organized matrix. A good balance in synthesis, degradation, and remodeling is necessary for matrix integrity maintenance. In addition, keratocytes also produce metalloproteinase inhibitor proteins, which seem essential to keep matrix integrity. Just in confirmation of this, our results show upregulation of genes involved in metalloproteinase inhibitor activity and endopeptidase inhibitor activity (Figure 6, Molecular Function) [19].

We also know about the next route that a large amount of Na + was absorbed in the protein digestion and absorption pathway, On the other hand, They examined ion transfer in the rat cornea and found Na+ is a major component

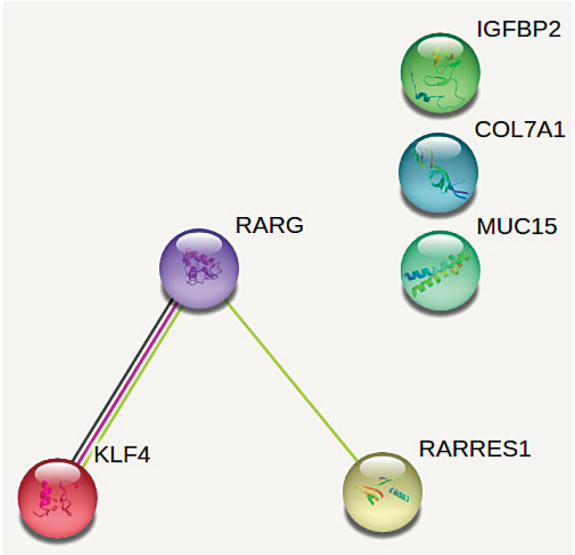


Figure 8: STRING network of down-regulated DEGs

of ion transport in the cornea [20].

Glycosphingolipids are a type of glycolipid expressed in all vertebrate cells and body fluids but expressed in the nervous system abundantly. Some studies show that Glycosphingolipids are located in large quantities in the cell surface microdomains and are effective in cell adhesion and mediate the transmission of signals. This may be the reason why this pathway is active in the optic nerve [21, 22].

About another pathway, Pyrimidine metabolism, previously reported that it is an active pathway in the optic nerve [23]. Also about what is in gene ontology biological process, flickering light leads to increased blood flow in the optic nerve head by 256 % nitric oxide regulates blood flow of optic nerve head and save normal vascular tone [24, 25]. And about the regulation of pH, explains electrogenic Na+/HCO cotransport in optic nerve cells and its role in regulating its pH [26].

The voltage-gated calcium channel is one of the ion-carrying channels found on the membrane surface of excitable cells such as neurons. Calcium ions are exchanged between intracellular and extracellular spaces through these channels. These channels are also present in optic nerve cells [27, 28].

Figure 6 also shows potassium channels in the biological process part. [29] has said about the activity of the electrogenic pump (Na + / K + -ATPase) in the optic nerve.

Conclusion

In this paper, we used strict methods to report a gene as DEG in tissues, and eventually reached a small number of DEGs (especially in the cornea), but traces of Enrichment analysis can be seen in other articles. Since we did not use the FDR method and set the adjusted P value to 0.05, we avoided false positives and we can consider the results valuable and correct.

Future works

We found genes that have different expressions in both optic nerve and corneal tissues, but we have not yet decided which gene is specific for which type of tissue, or we have not yet decided whether from a stem cell to tissue in vitro. When we reach the cornea, which genes should we target. We can select another independent database that includes, for example, samples of corneal tissue and any other tissue. Then check that each of the genes we obtained; have different expressions in the new database or not? In this way, we can finally reach the specific genes of the cornea.

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Footnotes and Financial Disclosures

Conflict of interest

The authors have no conflict of interest with the subject matter of the present manuscript.