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Differential Effects of Elevated Hydrostatic Pressure on Gene Expression and Protein Phosphorylation in Optic Nerve Head Astrocytes

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1. Introduction

1.1 Primary Open Angle Glaucoma (POAG)

Elevated intraocular pressure (IOP) is the most important risk factor in POAG. Typically affecting older adults, in POAG the IOP exceeds the level that is tolerated by that individual's optic nerve head (ONH). However, many individuals with clinical ocular hypertension do not exhibit glaucomatous changes in the optic disk; whereas, some individuals will develop glaucomatous changes at clinically normal IOP levels. These clinical findings indicate that individual variability in susceptibility of the ONH to IOP is an important factor in glaucomatous optic neuropathy. However, the molecular and cellular factors that may underlie variability in susceptibility of the ONH to elevated IOP have not been elucidated.

The target of the mechanical stress generated by elevated IOP is the lamina cribrosa in the ONH (Bellezza et al., 2003). In the glaucomatous ONH, compression, stretching, and remodeling of the cribriform plates of the lamina cribrosa occur. In many POAG patients, these elevated IOP related changes result in remodeling of the extracellular matrix (ECM), altering the quantity and composition of several ECM macromolecules that significantly affect the biomechanical properties of the tissue supporting the nerve fibers (Hernandez, 2000; Bellezza et al., 2003). Alterations of ECM components of the ONH in glaucoma perhaps sets the stage for further optic nerve damage from IOP during the progression of the disease.

1.2 POAG in African Americans

Epidemiological and genetic studies indicate that ethnic/genetic background plays an important role in susceptibility to POAG. POAG is more prevalent in Black Americans of African ancestry (AA) than in White Americans of European ancestry (CA), with reported frequencies of 3-4% in the AA population over the age of 40 years, as compared with approximately 1% in CA populations (Friedman et al., 2004). The disease is particularly frequent in Afro-Caribbean populations, with prevalence of 7% in Barbados and 8.8% in St. Lucia (Nemesure et al., 2001). On average AAs have increased duration (Quigley and Vitale, 1997) and progression of disease (Broman et al., 2008) compared to other populations. A positive family history of POAG is a major risk factor for the disease in AA (Leske et al.,

2008). The Advanced Glaucoma Intervention Study (AGIS), which compared the glaucoma outcomes in AA and CA patients, concluded that after failure of medical therapy, surgical trabeculectomy delayed progression of glaucoma more effectively in CA than in AA patients (Beck, 2003; Ederer et al., 2004). AAs have significantly larger disc areas, larger cup areas, larger cup-to-disc ratios and smaller neural rim area-to-disc area ratios compared with CAs (Varma et al., 1994; Quigley et al., 1999). A morphometric study determined that AAs have a larger total area of the lamina cribrosa and a greater number of pores than CAs (Dandona et al., 1990). The cellular and molecular bases for these anatomic differences and disease prevalences have not been explored.

Our studies focus on how astrocytes in the lamina cribrosa of the human optic nerve head contribute to glaucomatous optic neuropathy that is associated with elevated IOP. There is mounting evidence that astrocytes are responsible for many of the pathological changes in the glaucomatous ONH and cells isolated from donors of different ethnic backgrounds vary in their gene expression (Miao et al., 2008; Hernandez et al., 2008). Cellular explanations for these population-related differences may be realized using primary ONH astrocytes as a source of differing cellular phenotypes and an *in vitro* system to simulate elevated IOP.

1.3 Astrocytes in the ONH

Astrocytes are the major glial cell type in the non-myelinated human ONH and provide cellular support functions to the axons while interfacing between connective tissue surfaces and surrounding blood vessels. In the normal ONH, astrocytes are quiescent, terminally differentiated cells. In the lamina cribrosa, quiescent astrocytes form lamellae oriented perpendicular to the axons surrounding a core of fibroelastic extracellular matrix (Hernandez, 2000). Astrocytes supply energy substrates to axons in the optic nerve and maintain extracellular pH and ion homeostasis in the periaxonal space (Fields and Stevens-Graham, 2002). ONH astrocytes express ECM proteins such as laminin, and proteoglycans, as well as bone morphogenetic proteins (Zode et al., 2007; Wordinger et al., 2002) neurotrophins and receptors (Lambert et al., 2004; Yang et al., 2007). Several of these may serve as neuroprotective factors for retinal ganglion cells (RGC). In addition to astrocytes, other cell types exist in the lamina cribrosa of humans and non human primates, including microglia, vascular endothelia and the lamina cribrosa cell. Lamina cribrosa cells can be distinguished from astrocytes because they do not express glial fibrillary acidic protein (GFAP) and they do not express vascular or microglial markers (Hernandez et al., 1988; Kirwan et al., 2005; Hernandez et al., 1989).

1.4 Reactive ONH astrocytes in glaucoma

Adult, quiescent astrocytes become "reactive" after injury or disease and participate in formation of a glial scar, which does not support axonal survival or growth (Sofroniew, 2005; McGraw et al., 2001; Hatten et al., 1991). The major hallmarks of a reactive astrocyte are an enlarged cell body and a thick network of processes with increased expression of GFAP and vimentin (Hatten et al., 1991). Similarly, reactive astrocytes in the glaucomatous ONH are large rounded cells with many thick processes and express increased amounts of GFAP, vimentin and HSP27 (Hernandez et al., 2008). The astrocytes are motile and migrate either to the edge of the laminar plates or to inside the nerve bundles (Hernandez et al., 2008). In glaucoma, reactive astrocytes exhibit putative neurodestructive and neuroprotective cellular cascades in the ONH (Hernandez and Pena, 1997). Previous studies demonstrated that the genomic

responses of astrocytes in the glaucomatous ONH serve as the basis for these cascades (Hernandez et al., 2002). Damage to retinal ganglion cell axons and remodeling of the connective tissue plates in the lamina cribrosa appear to be mediated by astrocytes (Hernandez et al., 2008; Nickells, 2007). Reactive ONH astrocytes increase expression of various cell surface and extracellular matrix-related proteins such as laminin, tenascin C, and proteoglycans that play important roles in cell-cell recognition and in cell adhesion (Hernandez et al., 2002; Hernandez and Pena, 1997; Pena et al., 1999b). As might be expected there are also changes in signal transduction in reactive ONH astrocytes (Review (Hernandez et al., 2008)). For example, EGF receptors (Liu et al., 2006) and endothelin B receptors (Wang et al., 2006) are upregulated *in vitro* and *in vivo*. Finally differential gene expression analysis of human ONH astrocytes (Miao et al., 2008) (CA vs. AA) and astrocytes from AA compared to CA donors with glaucoma (Lukas et al., 2008) has revealed changes in multiple signaling systems that are also found in animal models of glaucoma (Johnson et al., 2007; Yang et al., 2007).

1.5 Susceptibility to glaucoma: Differential expression in the ONH astrocyte transcriptomes from glaucomatous AA and CA donors

Gene expression in primary cultures of ONH astrocytes obtained from age-matched normal and glaucomatous donors of CA and AA populations was done using Affymetrix GeneChip microarrays. Gene Ontology analysis and networks of interacting proteins were constructed using the BioGRID database (Stark et al., 2006). The differential gene expression data were distributed among three networks that include regulation of myosin, actin, and protein trafficking (Lukas et al., 2008). Remarkably, cultured glaucomatous astrocytes retain differential expression of genes that promote cell motility and migration, regulate cell adhesion, and are associated with structural tissue changes during neurodegeneration. Similar changes in gene expression were observed in glaucomatous optic nerve head tissues as assessed by immunohistochemistry (Lukas et al., 2008). Thus, the *in vitro* culture system “remembers” its origins from glaucomatous or control tissue. In these studies, the key differentially regulated genes included myosin light chain kinase (MYLK), TGF β receptor 2 (TGFBR2), Rho-family GTPase-2 (RAC2), and extracellular matrix protein, versican (VCAN). These differentially expressed components of integrated networks may reflect cellular and functional responses to chronic elevated IOP that are enhanced in the glaucomatous astrocytes from AA donors.

1.6 Model systems for glaucomatous astrocytes

One of the significant problems associated with work on glaucomatous astrocytes is that their availability is quite limited as the number of donors with glaucoma is much smaller than donors without eye disease. Therefore, we investigated methods to “transform” normal ONH astrocytes into a preeglaucoma or glaucoma-like phenotype. In principle, mechanical stress applied to the cells by way of physical contact (as in the ONH) should mimic the *in vivo* phenotype. Such stress can be done using a mechanical device in which the cells are attached to a deformable surface. Trabecular meshwork (Liton et al., 2005), lamina cribrosa (Kirwan et al., 2004), and smooth muscle cells (Feng et al., 1999) have been studied in such systems to mimic forces of mechanical stretch. Some attempts have been made with astrocytes as well (Neary et al., 2003). However, high force stretch is more often associated with traumatic astrocyte injury (Ni et al., 1997) rather than the lower forces associated with increased IOP in glaucoma. Thus, our *in vitro* IOP model employs elevated

hydrostatic pressure in an environmental chamber (Salvador-Silva et al., 2001) that compensates for gas composition and changes in media over time (Yang et al., 2004; Ricard et al., 2000). This model system has been used in a number of studies to study changes in bimolecular and cellular properties associated with this induced stress (Yang et al., 2004; Salvador-Silva et al., 2004; Salvador-Silva et al., 2001; Hernandez et al., 2000; Malone et al., 2007; Yang et al., 1993; Chen et al., 2009; Miao et al., 2010).

2. Results-gene expression studies in ONH astrocytes

2.1 Elevated hydrostatic pressure model system to simulate elevated IOP

We investigated the molecular bases of elevated hydrostatic pressure (HP) responses in primary cultures of ONH astrocytes derived from AA donors compared to CA donors. This model system simulates the mechanical stresses placed upon the optic nerve head by elevated IOP. Using high density microarrays we investigated global changes in gene expression in a cohort of three astrocyte cell lines from each group. We validated changes in expression induced by elevated HP in three to five additional AA and CA astrocyte lines using quantitative RT-PCR and/or Western Blotting. Using a global phosphoproteome approach, we also identified changes in protein phosphorylation associated with elevated HP. In both gene expression and proteome experiments, ONH astrocytes were subjected to elevated HP for periods of 3, 6, 24, and 48 hours. Control cells were cultured under ambient pressure (CP) for 6 or 48 hr. A custom made pressure chamber was used to subject cultured cells (6 well plates) to 60 mm (above atmospheric pressure) of HP for the desired periods of time. In order to compensate for potential changes in dissolved oxygen or pH, the gas mixture in the chamber was adjusted to 8% CO₂ and buffering capacity in the media increased (Yang et al., 2004). Under these conditions changes in dissolved oxygen and pH are negligible during the course of the experiment (Ricard et al., 2000). Messenger RNA and protein were extracted from the cells immediately after the experiment using standard published techniques (Lukas et al., 2008; Miao et al., 2010; Chen et al., 2009). Gene expression was measured using Illumina Human-6 beadchips following the manufacturer's protocols. These microarray chips have ~47,000 features representing ~31,000 human genes and expressed isoforms.

2.2 Microarray data analysis

To best utilize the unique features of Illumina BeadArray technology, we used the Bioconductor lumi package (Du et al., 2008; Lin et al., 2008; Du et al., 2007) to preprocess the raw data output by Illumina Beadstudio software. The data was preprocessed using variance stabilization transformation method (Lin et al., 2008) followed by quantile normalization. Probes with all samples "Absent" (lower or around background levels) were removed from further analysis to reduce false positives.

Two phases of analysis were done. First, to identify differentially expressed genes common to both the AA and CA groups, we applied routines implemented in limma package (Du et al., 2008) to fit linear models to the normalized expression values. The variance used in the t-score calculation was corrected by an empirical Bayesian method (Smyth, 2004) for better estimation with a small sample size. For each time point (3h, 6h, 24h and 48h), we compared all HP samples with the control samples, and defined the differentially expressed genes as fold-change higher than 1.3-fold ($p < 0.01$). After this filtering we found 352 genes differentially expressed at least at one time point. In order to reduce variations across different clinical samples, we first normalized the pressure samples at each time point

against the control sample from the same eye, then we standardized the normalized expression profile of each gene as one standard deviation and without centering (the fold-change direction is unchanged). Hierarchical clustering was first applied to get an overview of the cluster distribution, and then we defined initial clusters with visually selected cutoff. Using these initial sets, we did K-Means clustering resulting in 4 clusters that include 67, 137, 56 and 93 genes, respectively. The average changes in expression for each cluster as a function of time of HP treatment are shown in Figure 1. The cluster profile is defined as the average of all gene time profiles in the cluster (solid lines in Figure 1). Clusters 1 and 4 exhibit a decreasing trend of expression, while clusters 2 and 3 have increased expression as a function of time of exposure to elevated HP. The profiles of clusters 3 and 4 exhibit a flattening at the 3 and 6 hr times followed by a continued increase or decrease at 24 and 48 hr. These data suggest that ONH astrocytes respond to elevated HP in a dynamic fashion that involves a fast (3-6 hr) and slower (or protracted) response that follows at 24-48 hr. These responses involve genes in different signaling pathways that define a transition to new cellular phenotypes. As we will demonstrate in subsequent sections, there is overlap between the pressure-induced phenotype and glaucomatous cellular profiles that have been described previously (Hernandez et al., 2002; Nickells, 2007). Similarly, we found that extended HP treatment of ONH astrocytes (72-120 hr) induces further changes in cellular phenotype with respect to the expression of genes that affect cell motility (Miao et al., 2010) and cell morphology (Yang et al., 1993).

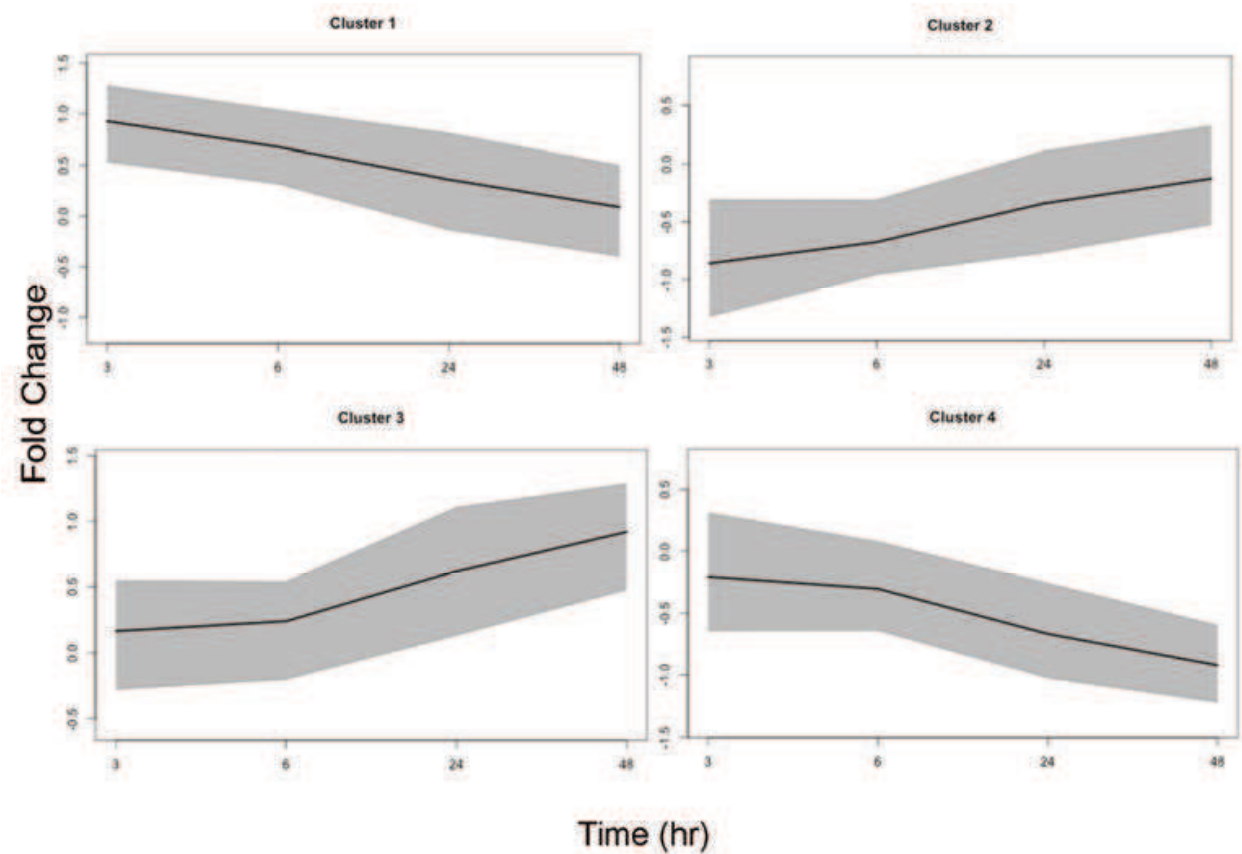


Fig. 1. **Time dependent expression of HP-induced gene clusters.** Solid lines are the fold-change (compared to cells at ambient pressure) average of all genes in the cluster while the gray areas indicate the 95% confidence interval of the averages.

2.3 HP-induced cellular processes in ONH astrocytes

For consistent comparison to the glaucomatous phenotype for ONH astrocytes (Nickells, 2007) we used the GeneGo suite to analyze the differential expression data for our elevated HP data. Using the gene clusters identified above, the genes were mapped onto existing canonical pathways that have been defined within GeneGo (Nikolsky et al., 2005). There are 650 such maps within the GeneGo library. The overrepresentation of genes within a pathway gives a p-value that estimates the significance of the mapped genes. The four top scoring maps for each cluster are summarized in Tables 1 and 2.

Cluster 1				
	Map	Cell Process	p-value	Objects
1	Estradiol Metabolism		0.00007	3/19
2	Regulation of Triiodothyronine and thyroxine signaling	Transcription	0.0054	2/31
3	Ligand dependent transcription of retinoid target genes	Transcription	0.0047	2/32
4	Androgen receptor nuclear signaling	Transcription	0.0094	2/64
Cluster 2				
1	Cell cycle-ESR1 regulation of G1/S transition	Response to hormone, extracellular stimulus	0.00001	6/32
2	BMP Signaling/ TGFβ inhibition	Cytokine mediated signaling	0.00033	3/33
3	Cell cycle regulation	Cell cycle	0.0063	3/29
4	Chemokines and cell adhesion	Cell Adhesion	0.0079	3/50

Table 1. Pathway analysis for Gene Cluster s 1 and 2 ranked by p-value. The number of objects refers to the enrichment of genes/total gene related objects in the map.

In the first two clusters, the activation of transcription and cytokine mediated signaling are among the most significant pathways. The transcriptional processes decline with increased time of HP suggesting that this is an early response that stabilizes to near control levels at 48 hr. As will be shown later this response also correlates with changes in protein phosphorylation. The other significant pathways in Clusters 1 and 2 are cytokine-related or other extracellular stimuli. These increase with time of HP treatment suggesting a transition to a phenotype associated with increased sensitivity to extracellular agents. In this regard, this cluster includes serotonin receptor 5B (Table 5), a receptor known to be upregulated by mechanical stress (Liang et al., 2006; Sanden et al., 2000). Cluster 3 genes are found in pathways leading to cytoskeleton remodeling, transport, and other signaling processes. ONH astrocytes undergo changes in morphology after being subjected to elevated HP (Yang et al., 1993) and up-regulation of cytoskeletal signaling processes is consistent with these properties. Moreover, altered cytoskeletal , transport, and cell adhesion gene expression is also found in populations of glaucomatous astrocytes (Lukas et al., 2008).

	Cluster 3			
1	Map Cell contraction mediated by G-protein coupled receptors	Cell Process Cytoskeleton	p-value 0.0019	Objects 3/15
2	Transport_RAB1A regulation pathway	ER-Golgi transport	0.0004	2/12
3	Cytoskeleton remodeling by Rho GTPases	Cytoskeleton	0.0053	4/23
4	Regulation of insulin pathway signaling	Receptor-mediated signaling	0.0050	2/42
	Cluster 4			
1	Transcription – Sin3 and NuRD in transcriptional regulation	Transcription	0.00059	3/38
3	G-protein receptor signaling via cyclic AMP pathways	Extracellular mediated signaling	0.0022	2/52
2	Regulation of lipid metabolism by transcription factor RXR via PPAR, RAR, and VDR pathways	Transcription	0.0075	2/30
4	Slit-Robo signaling in regulation of neuronal axon guidance	Development	0.0075	2/30

Table 2. **Pathway analysis of Cluster 3 and 4 ranked by p-value.** The number of objects refers to the enrichment of genes/total gene related objects in the map

Cluster 4 genes populate a distinct set of pathways in transcription, metabolism, and signal transduction. Inhibition of axonal guidance is altered under elevated HP due to secretion of Slit2/3 (Table 3) by astrocytes decreased with elevated HP over time. Further decreases in transcriptional pathways are evident in Cluster 4 with changes in the retinoid (RXR) based regulatory pathways. The latter involves changes in transcription through the post-translational modification of histones (Figure 2). Thus, besides the decrease in expression histones can undergo acetylation and methylation of lysine residues and phosphorylation of serine/threonine residues that alter chromatin structure and the transcription of specific DNA regions (Oki et al., 2007; Ito, 2007). As shown below we detected phosphorylation of lysine methyltransferase enzymes in ONH astrocytes at early time points of treatment with elevated HP. This is particularly interesting because there are few reports of epigenetic changes being induced by mechanical stress (Illi et al., 2005) and none by elevated HP. Thus, the transcriptome of ONH astrocytes may be undergoing reprogramming to accommodate this presence of this stress.

Other pathways represented in Cluster 4 genes that are altered by elevated HP include cyclic-AMP signaling. Our previous studies of short term elevated HP (30 min – 3 hr) treatment of ONH astrocytes revealed that cyclic-AMP signaling is differentially affected in astrocytes from African American donors (Chen et al., 2009). Upregulation of adenylate cyclases and parathyroid hormone-like hormone (PTH1H) that activates G-protein signaling to adenylate cyclases were detected (Chen et al., 2009). In the current work with longer term (24-48 hr) treatment both AA and CA astrocytes down regulate phosphodiesterases (PDE3A, PDE7B (Table 3.) which may result in higher levels of cyclic nucleotides and increased activity of transcription factors such as CREB that are activated by cyclic AMP-dependent phosphorylation (Johannessen et al., 2004; Delghandi et al., 2005).

Symbol	Description	Cluster 1	HP3	HP6	HP24	HP48
IL6	interleukin 6 (interferon, beta 2)		2.22	1.41	-1.15	-1.67
TAF5L	TAF5-like RNA polymerase II, p300/CBP-associated factor (PCAF)		1.59	1.24	1.44	1.38
PAG1	phosphoprotein associated with glycosphingolipid microdomains 1		1.44	1.11	-1.11	1.06
WNT5B	wingless-type MMTV integration site family, member 5B		1.38	1.26	1.22	1.08
ELF4	E74-like factor 4 (ets domain transcription factor)		1.37	1.15	1.10	-1.05
ITCH	itchy E3 ubiquitin protein ligase homolog		1.35	1.35	1.18	1.10
SULT1A3	sulfotransferase family, cytosolic, 1A 3		1.33	1.18	1.24	1.03
NCOR2	nuclear receptor corepressor 2		1.18	1.34	-1.02	1.02
Cluster 2						
FOS	FBJ osteosarcoma viral oncogene homolog		-1.21	-1.74	-1.58	-1.44
MYC	v-myc myelocytomatosis viral oncogene homolog		-1.39	-1.37	-1.08	-1.03
SKP2	S-phase kinase-associated protein 2 (p45)		-1.30	-1.09	-1.00	1.03
CREB1	cAMP responsive element binding protein 1		-1.32	-1.42	-1.21	-1.16
OSMR	oncostatin M receptor		-1.16	-1.23	-1.53	-1.14
ITGA6	integrin, alpha 6		-1.39	-1.34	-1.76	-1.13
PITX1	paired-like homeodomain 1		-1.44	-1.25	-1.27	-1.37
BMP4	bone morphogenetic protein 4		-1.79	-1.35	1.04	1.06
SMAD5	SMAD family member 5		-1.34	-1.04	-1.00	-1.22
TGIF1	TGFB-induced factor homeobox 1		-1.10	-1.37	1.05	1.19
COL4A4	collagen, type IV, alpha 4		-1.56	-1.63	-1.32	-1.88
CFL2	cofilin 2		-1.12	-1.67	1.00	1.24
HTR2B	5-hydroxytryptamine receptor 2B		-1.61	-1.69	1.05	1.16
Cluster 3						
MYL9	myosin, light chain 9, regulatory		1.05	1.04	1.11	1.42
PIK3IP1	phosphoinositide-3-kinase interacting protein 1		-1.04	1.22	1.04	1.39
IRS2	insulin receptor substrate 2		-1.04	-1.35	1.36	1.43
MYO1D	myosin ID		1.06	1.12	1.34	1.22
NAPA	N-ethylmaleimide-sensitive factor attachment protein, alpha		-1.02	-1.02	1.21	1.31
RUSC2	RUN and SH3 domain containing 2		1.17	1.03	1.39	1.23
RPS6KB2	ribosomal protein S6 kinase, 70kDa		1.10	1.17	1.15	1.39
PKN1	protein kinase N1		-1.28	-1.08	1.03	1.32
Cluster 4						
PPARG	peroxisome proliferator-activated receptor gamma		-1.46	-1.28	-1.46	-1.28
PDE7B	phosphodiesterase 7B		-1.27	-1.20	-2.09	-2.06
HIST1H2BG	histone cluster 1, H2bg		-1.26	-1.19	-1.50	-1.31
THRA	thyroid hormone receptor, alpha		-1.24	-1.23	-1.30	-1.56
HIST1H2BD	histone cluster 1, H2bd		-1.23	-1.34	-1.63	-1.77
HIST1H4H	histone cluster 1, H4h		-1.18	-1.31	-1.38	-1.50

HIST1H4E	histone cluster 1, H4e	-1.16	-1.17	-1.33	-1.54
RARB	retinoic acid receptor, beta	-1.14	1.03	-1.39	-1.28
HIST2H4A	histone cluster 2, H4a	-1.11	-1.29	-1.45	-1.48
SLIT2	slit homolog 2 (Drosophila)	-1.08	-1.07	-1.22	-1.56
TGFBR2	transforming growth factor, beta receptor	-1.07	-1.08	-1.26	-1.37
HIST1H3D	histone cluster 1, H3d	-1.05	-1.25	-1.52	-1.49
HIST2H2AA3	histone cluster 2, H2aa3	1.04	-1.01	-1.31	-1.45
PDE3A	phosphodiesterase 3A, cGMP-inhibited	1.00	-1.62	-1.24	-1.54

Table 3. Selected genes from clusters 1-4 that are found in relevant signaling pathway maps (Tables 1 and 2). Fold change in expression (from controls) is indicated at each time point (HP3, HP6, HP24, HP48).

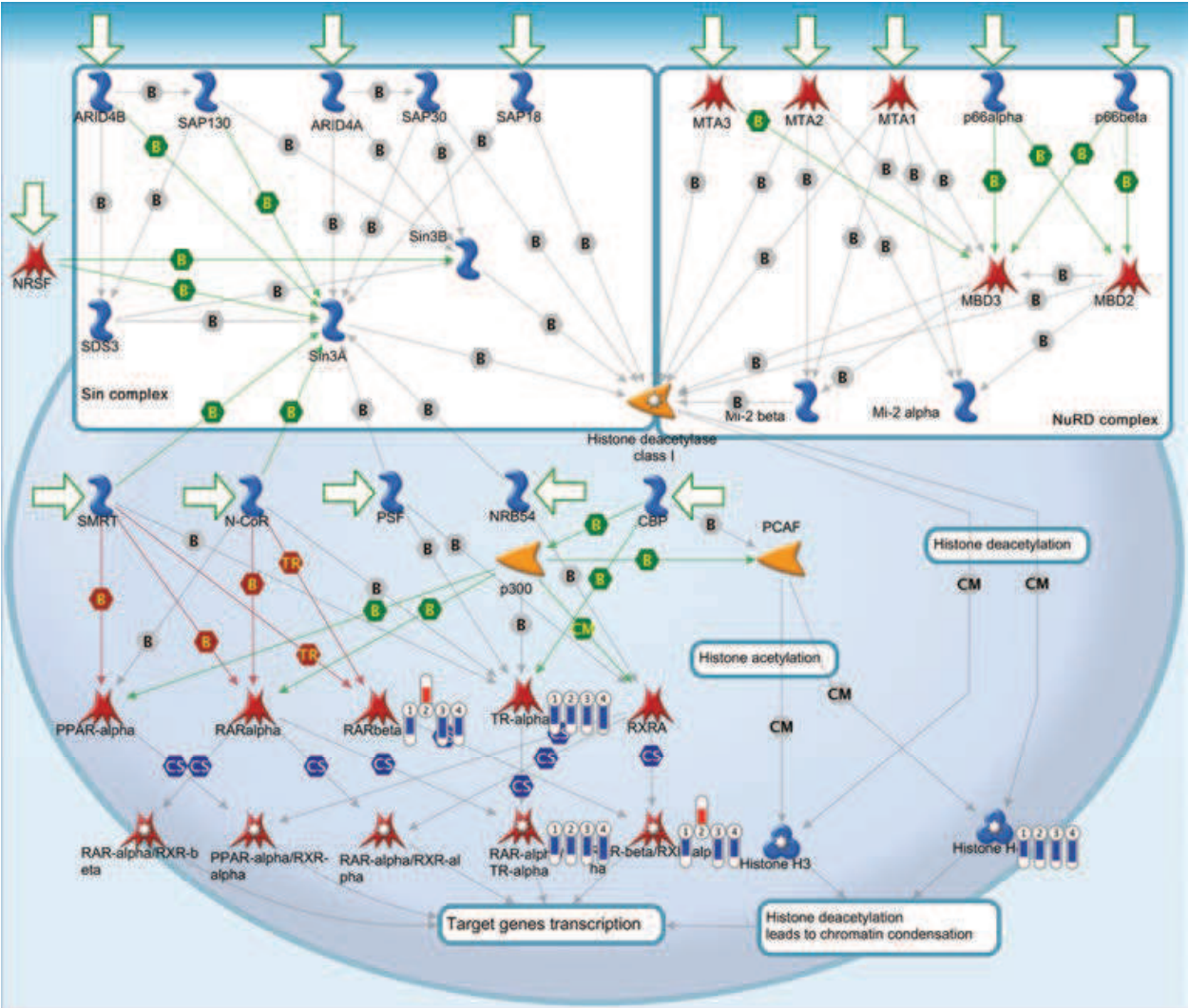


Fig. 2. Pathway map for differentially expressed transcription factors and histones in ONH astrocytes subjected to elevated HP. The genes with altered expression have a row of four “thermometers” adjacent to them that indicate the fold changes with time of exposure to elevated HP. (Blue thermometers are decreased fold-change, while red are increased fold-change.

2.5 Differential gene expression in ONH astrocytes from African American compared to Caucasian donors after treatment with elevated HP

Using the same gene selection and filtering criteria described above for the combined AA and CA data sets, we performed a cross comparison of gene expression at the same time points for genes differentially expressed by AA astrocytes. The 63 genes in Figure 3 were selected by satisfying these criteria: 1. a fold-change > 1.3, p-value < 0.01 of HP-AA vs. Ctrl at least at one time point, 2. a p-value < 0.01 of normalized HP-AA vs. normalized HP-CA at least at one time point (each time point was normalized against control).

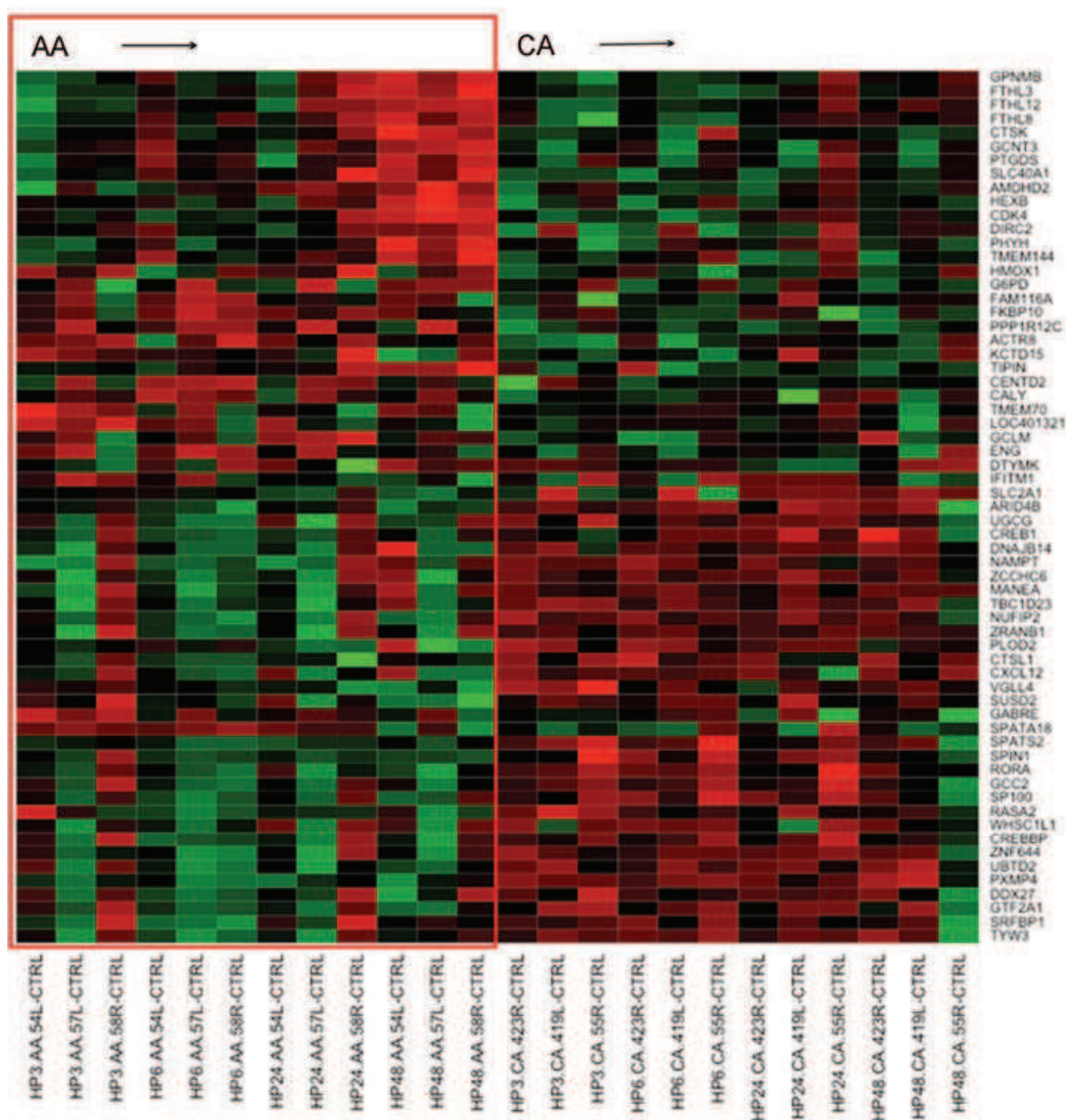


Fig. 3. Heat map of gene expression within individual ONH cell lines as a function of time of elevated HP treatment. The red, black and green colors represent higher than average, close to average and lower than average expression of the particular gene, respectively. The genes corresponding to each row of the heat map are on the right hand side of the picture.

To cluster the genes, we first standardized the normalized expression profile of each gene to one standard deviation and without centering (to keep the fold-change direction unchanged), then applied hierarchical cluster to get an overview of the cluster distribution. Based on the hierarchical clustering results, we defined initial clusters with visually selected cutoff value (5.5 for our case). Using these initial clusters, we did K-Means clustering. A total of 6 clusters were obtained. Two representative gene expression cluster patterns over the time course are shown in Figure 4. The early response genes (Figure 4A) increased significantly in AA (in red dotted line) after 3h exposure to HP compared to CA (black solid line). The late response genes (Figure 4B) increased significantly in AA (red dotted line) after 48h exposure to HP compared to CA (black solid line).

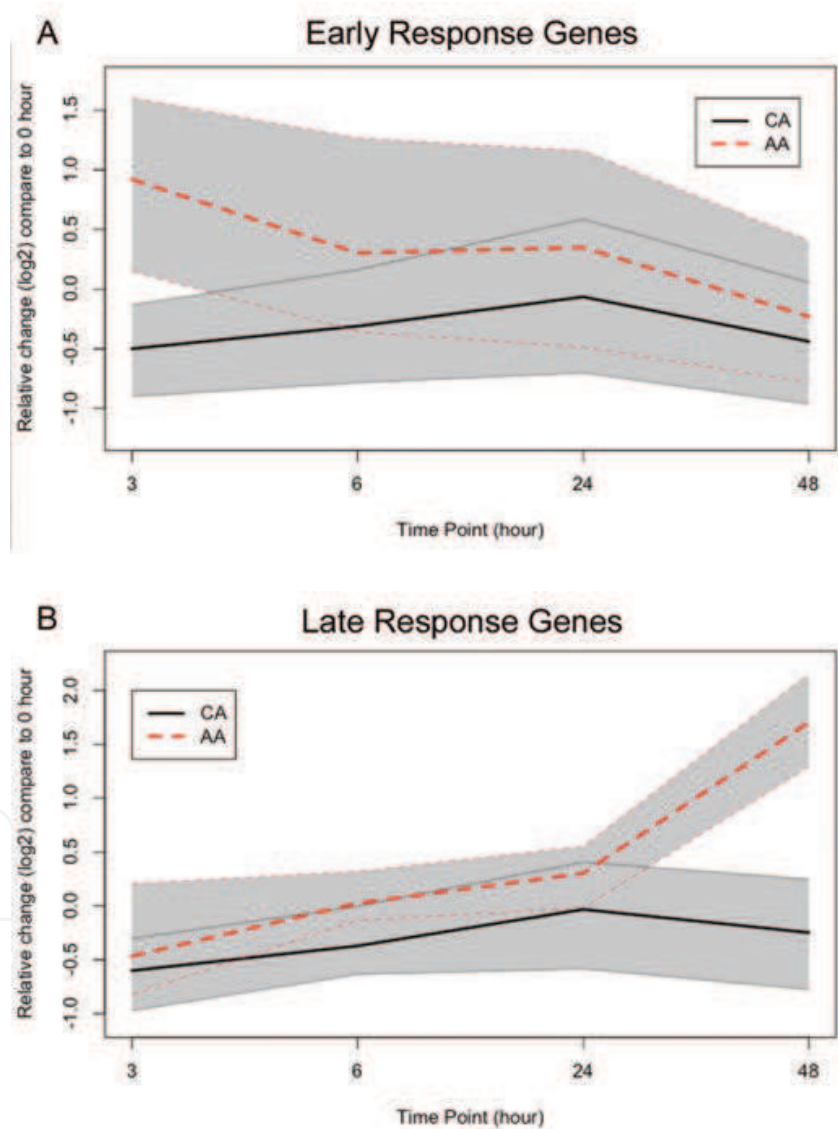


Fig. 4. **Altered gene expression in AA ONH astrocytes after elevated HP as a function of time.** A. Early response genes are those that differentially changed in AA compared to CA astrocytes after 3-6 hr of HP. B. Late response genes changed in AA or CA astrocytes at 24-48 hr of HP.

2.6 Validation of gene expression changes in ONH astrocytes subjected to elevated HP

Gene validation experiments were done primarily on the genes differentially expressed in the AA compared to CA ONH astrocytes as these were most likely to define some of the differences in sensitivity of AA cells to elevated HP. For validation, we tested GPNMB, CTSK, GCLM, HBEGF, and PLOD2. GPNMB is a late response gene. It has a requisite mutation (Anderson et al., 2008) in the hereditary glaucoma mouse model DBA-2J and is differentially expressed in ONH astrocytes in a primate ocular hypertension model (Kompass et al., 2008). Increased mRNA levels of GPNMB in AA correlate with the length of HP exposure (Figure 5A). Another late HP response gene is CTSK which is a lysosomal cysteine proteinase with strong degradative activity against the extracellular matrix and is suggested to play a role in tumor invasiveness. CTSK was significantly upregulated in AA

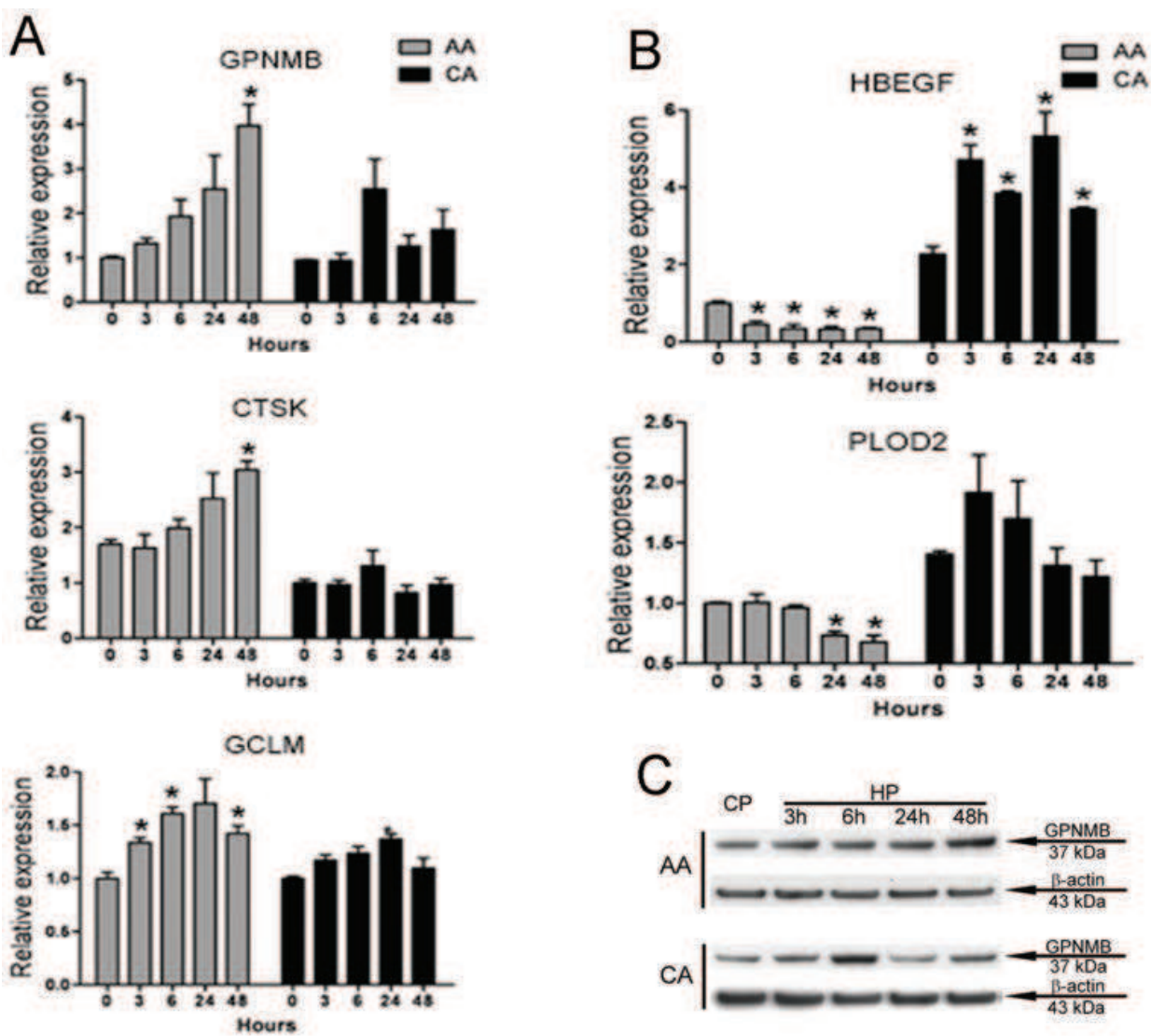


Fig. 5. Validation experiments on selected genes from ONH astrocytes treated with elevated HP. A. Quantitative real time PCR (qRT-PCR) of genes upregulated in AA astrocytes. B. qRT-PCR of Genes down regulated in ONH astrocytes. C. Western blot of GPNMB expression in ONH astrocytes.

astrocytes after 48 hr exposure to HP (Figure 5A). Two down regulated genes in the AA astrocytes compared to CA are HBEGF and PLOD2. In earlier work we found that HBEGF and PLOD2 were expressed significantly lower in native AA astrocytes compared to CA (Miao et al., 2008). When exposed to HP, HBEGF expression was further downregulated in AA, while upregulated in CA astrocytes (Figure 5B). PLOD2 encodes an enzyme which catalyzes the hydroxylation of lysyl residues in collagen-like peptides. It is important for the stability of intermolecular crosslinks in basement membranes. These data support our idea that the two populations of ONH astrocytes respond differently to elevated hydrostatic pressure with respect to changes in gene expression. Some responses may be due to intrinsically lower expression in the AA astrocyte population that is enhanced by elevated HP, while others appear to be specifically up-regulated in these cells.

2.7 Phosphoproteome profiling of ONH astrocytes subjected to elevated HP

Global phosphoproteome profiling was done using standard affinity-based methods to trap phosphopeptides coupled to high performance liquid chromatography-mass spectrometry (LC-MS) for detection and identification similar to our previous studies of changes in protein phosphorylation in the retina following optic nerve crush (Lukas et al., 2009). The strategy here was to determine whether changes in gene expression can be correlated with changes in protein phosphorylation in ONH astrocytes subjected to elevated HP. These studies were done on two pairs of ONH astrocytes- one pair from AA donors and the other

<i>Time of HP (hr)</i>	<i>GO category</i>	<i>p-value</i>	<i>#phosphoproteins</i>
0	Nuclear	0.0091	25
	Phosphorylation	0.029	6
	Cell Cycle	0.045	7
	Cytoskeleton	0.172	3
3	Nuclear	0.0025	25
	Phosphorylation	0.49	3
	Cell Cycle	0.04	5
	Cytoskeleton	0.02	8
6	Nuclear	0.0012	18
	Cell Cycle	0.04	5
	Cytoskeleton	0.02	6
	Phosphorylation	0.083	4
24	Nuclear	0.0013	19
	Cell cycle	0.004	7
	Cytoskeleton	0.029	6
	Phosphorylation	0.0085	6
48	Nuclear	0.231	12
	Cytoskeleton	0.003	8
	Cell cycle	0.47	2
	Phosphorylation	NS	0

Table 4. Gene Ontology categories enriched in phosphoproteins in AA and CA astrocytes at 0 (untreated), 3, 6, 24 and 48 hr of elevated HP treatment of ONH astrocytes.

from CA donors. As with the initial gene expression analysis, we combined all of the data at each time point to generate lists of the detected phosphoproteins. This was not a quantitative study, but rather a sampling of the phosphoproteome at each time point. This unbiased LC-MS method detects the most abundant phosphopeptides in the sample resulting in a limited survey of phosphoproteins. Phosphoproteome data were obtained at 3, 6, 12, and 24hr of elevated HP. These sets were individually submitted to Gene Ontology analysis using the GoMiner program (Zeeberg et al., 2003). During the time course of elevated HP, the number of detected nuclear and cell-cycle related phosphoproteins decreases and the cytoskeletal-associated phosphoproteins increase so as to make the differential enrichment significant (Table 4).

HP 3- 6hr	
Accession #	Nuclear or Cell cycle Phosphoproteins
IPI00448121	CDC7 Cell division cycle 7-related protein kinase
IPI00855998	CENPF Centromere protein F
IPI00103595	CEP350 Centrosome-associated protein 350
IPI00297851	CHD1 chromodomain helicase DNA binding protein 1
IPI00009724	EFCAB6 EF-hand calcium-binding domain-containing protein 6
IPI00015526	EHMT1 Histone-lysine N-methyltransferase, H3 lysine-9 specific 5
IPI00171903	HNRPM Isoform 1 of Heterogeneous nuclear ribonucleoprotein M
IPI00456887	HNRPUL2 Heterogeneous nuclear ribonucleoprotein U-like protein 2
IPI00299904	MCM7 Isoform 1 of DNA replication licensing factor MCM7
IPI00332499	NASP nuclear autoantigenic sperm protein isoform 1
IPI00470429	PARK2 Isoform 5 of E3 ubiquitin-protein ligase parkin
IPI00031627	POLR2A DNA-directed RNA polymerase II subunit RPB1
IPI00783392	RB1CC1 RB1-inducible coiled-coil protein 1
IPI00024710	RGS10 Regulator of G-protein signaling 10
IPI00004312	STAT2 Signal transducer and activator of transcription 2
IPI00044681	UHRF2 Isoform 1 of E3 ubiquitin-protein ligase UHRF2
IPI00783017	ZEB2 Zinc finger E-box-binding homeobox 2
IPI00003003	ZNF385D Zinc finger protein 385D
IPI00022177	ZNF559 Zinc finger protein 559
IPI00166972	ZNF608 Isoform 1 of Zinc finger protein 608
HP 24-48 hr	
IPI00747060	ANKRD45 Isoform 1 of Ankyrin repeat domain-containing protein 45
IPI00640320	CDC25C Isoform 2 of M-phase inducer phosphatase 3
IPI00009724	EFCAB6 EF-hand calcium-binding domain-containing protein 6
IPI00410079	FAM82C Isoform 1 of Protein FAM82C
IPI00171903	HNRPM Isoform 1 of Heterogeneous nuclear ribonucleoprotein M
IPI00307733	SETD2 Isoform 1 of Histone-lysine N-methyltransferase SETD2
IPI00025158	STAG1 Cohesin subunit SA-1
IPI00749005	SYNE1 1011 kDa protein
IPI00170921	TIGD7 Isoform 1 of Tigger transposable element-derived protein 7
IPI00217407	UBR2 Isoform 4 of E3 ubiquitin-protein ligase UBR2
IPI00796117	ZMYND11 BS69 variant 1
IPI00298731	PPP1R10 Serine/threonine-protein phosphatase 1 regulatory subunit 10

Table 5. Selected phosphoproteins found at early (3-6 hr) and late (24-48 hr) times of elevated HP in ONH astrocytes.

Summarized in Tables 5 and 6 are selected phosphoproteins identified in the early (3-6 hr) and late (24-48 hr) phases of elevated HP. Within the nuclear/cell cycle groups of phosphoproteins we find transcription factors, nuclear riboproteins, and ubiquitin ligases. Also notable are histone methyl transferase proteins that may be functioning to reprogram the ONH astrocyte gene expression in response to the stress induced by elevated HP. Phosphorylation of these proteins may function to activate epigenetic reprogramming of the ONH astrocyte in response to the stress induced by elevated HP.

HP 3-6 hr	Cytoskeletal Phosphoproteins
Accession#	Name
IPI00472779	ANK3 Ankyrin-3
IPI00011219	ARHGAP24 Rho GTPase-activating protein 24
IPI00008756	DST Dystonin Isoform 1
IPI00306929	MYO18A Isoform 2 of Myosin-XVIIIa
IPI00479962	MYO5A Myosin-Va
IPI00307155	ROCK2 Rho-associated protein kinase 2
IPI00289639	VILL Isoform 1 of Villin-like protein
HP 24-48 hr	
IPI00010448	ARHGAP24 Rho GTPase-activating protein 24
IPI00457243	ARHGEF5 rho guanine nucleotide exchange factor 5
IPI00792788	DOCK5 Isoform 1 of Dedicator of cytokinesis protein 5
IPI00216408	DOCK9 Isoform 1 of Dedicator of cytokinesis protein 9
IPI00642259	DST Dystonin
IPI00260090	ELMO1/2 Engulfment and cell motility proteins
IPI00452247	KIF16B Isoform 2 of Kinesin-like motor protein C20orf23
IPI00848334	KIF2A Isoform 4 of Kinesin-like protein KIF2A
IPI00306929	MYO18A Isoform 2 of Myosin-XVIIIa
IPI00479962	MYO5A Myosin-Va
IPI00303335	NEB Nebulin
IPI00071509	PKP1 Isoform 2 of Plakophilin-1

Table 6. Selected cytoskeletal phosphoproteins identified at early (3-6 hr) and late (24-48) times of elevated HP in ONH astrocytes.

Within the cytoskeletal group were several proteins associated with focal adhesions (Table 6). These include for example, DOCK5/9 and ELMO1/2. ELMO was detected at the 3hr HP time point and the detected phosphopeptide, RIAFDAESEPNNSSGpSMEKR, corresponds to residues 329-348 of ELMO1. ELMO1 interacts with DOCK-180 and other proteins in a large complex found at focal adhesions (Beausoleil et al., 2006). Specific antiphosphopeptide antibodies to ELMO1 were not available, but there are phosphorylation site antibodies to p130CAS, another known phosphoprotein in the DOCK protein complex (Menniti et al., 2006). p130CAS is known to become highly phosphorylated in response to mechanical stress (Sawada et al., 2006; Geiger, 2006). We found that p130CAS phosphorylation increases with HP, particularly at the 3 and 6 hr time points in AA astrocytes and at 24-48 hr in CA astrocytes (Figure 6). Thus, the AA astrocytes respond earlier to elevated HP than the CA astrocytes with respect to p130CAS phosphorylation. Phosphorylation of p130CAS and ELMO may modulate the dynamics of focal adhesions. To support this idea, we found that

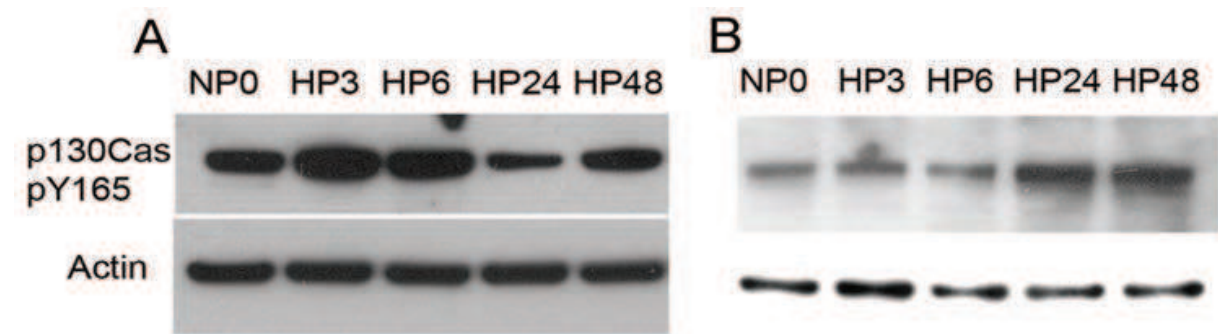


Fig. 6. Phosphorylation of p130CAS in A. AA and B. CA astrocytes . NP indicates cells at ambient pressure, while HP3-48 are cells exposed to elevated HP for those times. Antibodies specific for pY165 in p130CAS were used to probe Western Blots of cell extracts.

phosphorylation of focal adhesion kinase (FAK) was increased in CA astrocytes at 24-48 hr of elevated HP (Figure 7). Although FAK was phosphorylated in AA cells, its phosphorylation level was constant at each time point during elevated HP treatment (not shown).

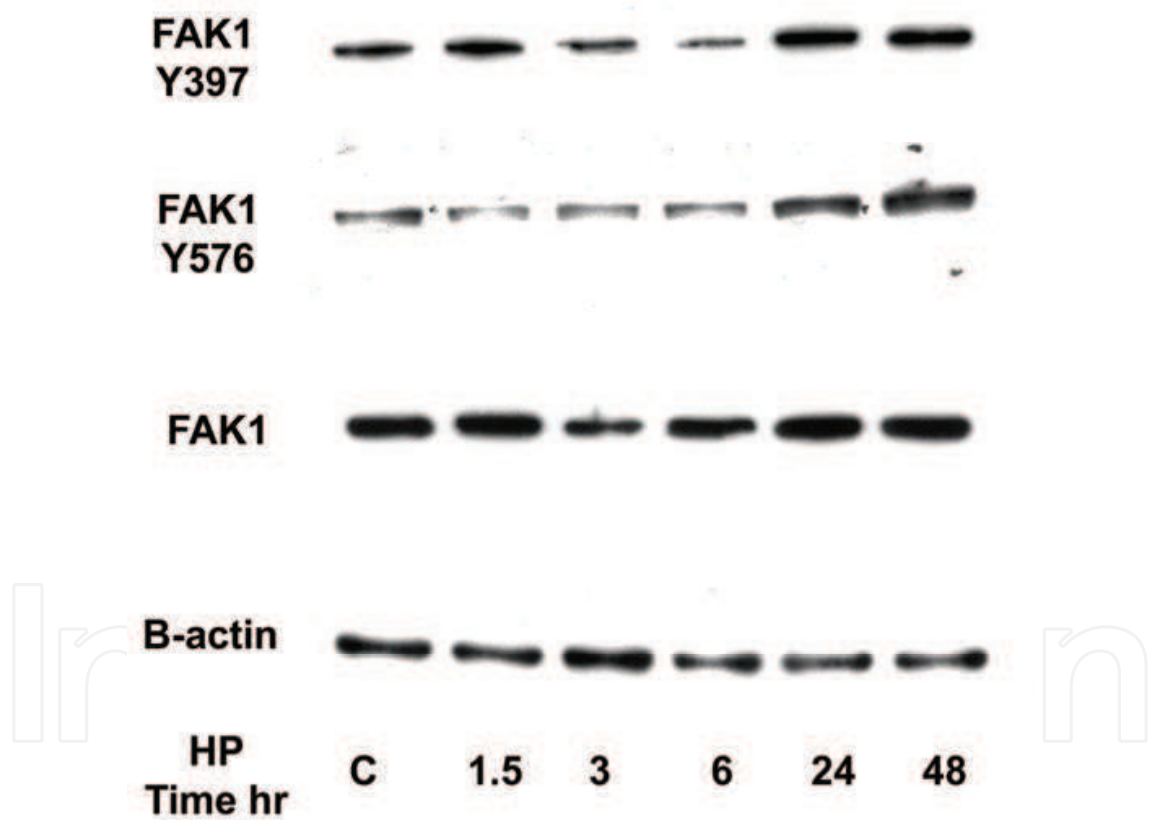


Fig. 7. Phosphorylation of focal adhesion kinase (FAK) in CA ONH astrocytes exposed to elevated HP. Antibodies specific to FAK phosphorylated at Y397 and Y576 were used to probe Western Blots of cell extracts.

2.8 Correlations of gene expression and protein phosphorylation in ONH astrocytes
The pattern of cytoskeletal protein phosphorylation is consistent with the differences in gene expression found in the late (24-48 hr) time of elevated HP treatment (Tables 1-3). Similarly, the pattern of protein phosphorylation at the early (3-6 hr) time of HP treatment is

consistent with the transcriptional activity associated with this phase (Tables 1-3). Thus, the elevated HP system provides a potential “preglaucoma” state in ONH astrocytes because differential changes in expression of genes in the TGF β pathways and cytoskeletal regulation are found in glaucomatous AA and CA astrocytes and in the glaucomatous ONH (Lukas et al., 2008). As shown earlier, elevated HP increases the expression of GPNMB protein in AA astrocytes compared to CA astrocytes (Figure 5C) consistent with the gene expression data. GPNMB is thought to function as a cell adhesion protein that connects the extracellular environment to downstream cellular signaling linked to gene transcription via TGF β pathways that modulates the synthesis of extracellular matrix proteins in ONH astrocytes (Fuchshofer et al., 2005). Therefore, these pathways may be larger contributors to changes in cell adhesion in AA astrocytes compared to CA. On the other hand, the activation of focal adhesions complexes is linked to cytoskeletal remodeling and transcription through multiple pathways (Koyama et al., 2000). Thus, in signal transduction and gene expression, AA and CA astrocytes exhibit differential responses to elevated HP that can produce altered cell adhesion and morphology.

3. Conclusions

The types of experiments described in this report fall into the realm of systems biology. The guiding hypothesis is that subjecting a system such as cultured ONH astrocytes, to a stressor (elevated HP) will cause changes in gene expression and protein phosphorylation in a fashion that impacts multiple signaling networks and processes. We found that, indeed, elevated HP induces changes in gene expression patterns that are setting up new cellular phenotypes. Thus, the system is a model for what elevated IOP may induce in ONH astrocytes *in vivo*. However, the biological and mechanical properties of the lamina cribrosa are complex, defined by contributions from astrocytes, lamina cribrosa cells and blood vessels embedded in a three-dimensional extracellular matrix. Thus, astrocytes and lamina cribrosa cells respond not only to external stimuli such as IOP but to the stiffness of the matrix within which they reside (Bellezza et al., 2003). Therefore, a cultured cell system is not a perfect model. However, both *in vitro* and *in vivo* cells continuously sample their mechanical microenvironment by exerting internally generated tensile forces on the surrounding matrix and adjust their phenotype in a cell- and tissue-specific manner. These sorts of responses are clearly evident in the hydrostatic pressure model used herein. Moreover, the parallels between model and the glaucomatous optic nerve head are converging on the same cellular signaling systems and alterations in gene expression. However, additional validation studies that examine gene expression in the optic nerve head of control and glaucomatous tissues will be needed. These sorts of studies have already been done to validate selected unregulated genes such as the transcription factors Fos and Jun (Hashimoto et al., 2005), cell adhesion molecules (Kobayashi et al., 1997; Ricard et al., 1999), extracellular matrix (Pena et al., 1999b; Pena et al., 1998), TGF β (Pena et al., 1999a) and TGF β receptors (Lukas et al., 2008).

In the same realm, changes in protein phosphorylation may define dynamic cellular phenotypes which have characteristics of different cellular populations. The few differences we uncovered in protein phosphorylation of cytoskeletal proteins between CA and AA astrocytes after treatment with elevated IOP are likely just a small sampling of the entire signaling system. However, one of the exciting prospects revealed in this study is that elevated IOP might be inducing epigenetic changes in ONH astrocytes that are responsible,

in part, for altering gene transcription programs. This opens up unexplored areas for future glaucoma research.

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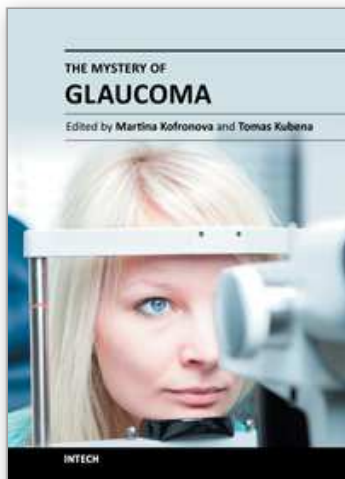
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Since long ago scientists have been trying hard to show up the core of glaucoma. To its understanding we needed to penetrate gradually to its molecular level. The newest pieces of knowledge about the molecular biology of glaucoma are presented in the first section. The second section deals with the clinical problems of glaucoma. Ophthalmologists and other medical staff may find here more important understandings for doing their work. What would our investigation be for, if not owing to the people's benefit? The third section is full of new perspectives on glaucoma. After all, everybody believes and relies – more or less – on bits of hopes of a better future. Just let us engage in the mystery of glaucoma, to learn how to cure it even to prevent suffering from it. Each information in this book is an item of great importance as a precious stone behind which genuine, through and honest piece of work should be observed.

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