Abstract

Objective: The retina is subjected to tractional forces in various conditions. As the predominant glial element in the retina, Müller cells are active players in all forms of retinal injury and disease. In this study, we aim to identify patterns of gene expression changes induced by cyclic mechanical stretching in Müller cells.

Methods: Rat Müller cells were seeded onto flexible bottom culture plates and subjected to a cyclic stretching regimen of 15% equibiaxial stretching for 1 and 24 h. RNA was extracted and amplified, labeled, and hybridized to rat genome microarrays. The expression profiles were analyzed using GeneSpring software, and gene ontology analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to select, annotate, and visualize genes by function and pathway. The selected genes of interest were further validated by Quantitative Real-time PCR (qPCR).

Results: Microarray data analysis showed that at 1 and 24 h, the expression of 532 and 991 genes in the Müller cells significantly (t-test, \( p < 0.05 \)) differed between the mechanically stretched and unstretched groups. Of these genes, 56 genes at 1 h and 62 genes at 24 h showed more than a twofold change in expression. Several genes related to response to stimulus (e.g., Egr2, Il6), cell proliferation (e.g., Areg, Atf3), tissue remodeling (e.g., PVR, Loxl2), and vasculogenesis (e.g., Epha2, Nr1) were selected and validated by qPCR. KEGG pathway analysis showed significant changes in MAPK signaling at both time points.

Conclusions: Cyclic mechanical strain induces extensive changes in the gene expression in Müller cells through multiple molecular pathways. These results indicate the complex mechanoresponsive nature of Müller cells, and they provide novel insights into possible molecular mechanisms that would account for many retinal diseases in which the retina is often subjected to mechanical forces, such as pathological myopia and proliferative vitreoretinopathy.

Introduction

The retina, which is responsible for encoding and processing visual stimulus, is subjected to tractional forces in various conditions. For instance, pathological myopia, which is one of the leading causes of blindness, is characterized by excessive and progressive elongation of the eyeball with concomitant degenerative changes in the posterior segment of the eye [1,2]. During the progressive distension of the posterior pole, the retina is overstretched, as a result of which retinal remodeling occurs. Moreover, mechanical stretching of the retina can also be observed over the course of posterior vitreous detachment [3], proliferative vitreoretinopathy, and so on. However, the cellular and molecular effects of mechanical stretching of the retina are relatively unexplored, and therefore, further research is required in this regard.

As the predominant glial element in the sensory retina, Müller cells are responsible for the homeostatic and metabolic support of retinal neurons, and they are active players in virtually all forms of retinal injury and disease [4,5,6]. Moreover, structurally, Müller cells span the entire retinal thickness, extending from the inner to the outer limiting membranes, with cell bodies located in the inner nuclear layer and lateral processes expanding into the plexiform layers of the tissue [6]. Because of this unique morphology, Müller cells can sense even minute changes in the retinal structure because of the mechanical stretching of their long processes or side branches. Thus, it is reasonable to infer that Müller cells also participate in ocular diseases where the retina is overstretched. In fact, a recent study confirmed that they were sensitive and responsive to tissue stretching [7]. However, the molecular effects of mechanical stretching on Müller cells remain unclear.

In this study, we aim to investigate the genome regulation of Müller cells under mechanical stretching in detail; this may provide clues to understanding the molecular mechanisms that would account for many retinal diseases in which the retina is often subjected to mechanical forces.
Results

Identification of Differentially Expressed Genes

Differential gene expression analysis showed that at 1 and 24 h, the expression of 532 and 991 genes significantly (t-test, p<0.05) changed between the mechanically stretched and the control groups (Tables S1 and S2). Of these, at 1 h, 56 genes, with 48 genes up and 8 genes down, showed more than a twofold change in expression (Table 1). At 24 h, 62 genes, with 16 genes up and 46 genes down, showed more than a twofold change in expression (Table 2). Subsequent analysis focused on these genes that showed a more than twofold change in expression (which was considered significant).

To visualize gene expression profiling at each time point, a hierarchical clustering analysis was carried out (Figure 1). The mechanically stretched and control cell cultures clustered independently in two separate primary branches of the dendrogram at both 1 and 24 h, indicating that Müller cells were responsive to stretching. More genes were up regulated at 1 h than at 24 h. To better demonstrate the process of identifying significant genes, volcano plots were also presented based on the microarray result (Figure 2). The red dots represent selected differentially expressed genes with p<0.05 and more than twofold change, most of which are listed in Tables 1 and 2.

Functional Gene Categories Regulated by Mechanical Stretching

As summarized in Table S3, after mechanical stretching for 1 h, 6 molecular functions, 5 cellular components, and 99 biological processes were significantly upregulated. These biological processes included cellular response to stimulus, multicellular organismal development, anatomical structure formation involved in morphogenesis, cell development, cell death, biosynthetic process, cell motion, cell proliferation, tissue remodeling, positive regulation of anti-apoptosis, positive regulation of metabolic process, and positive regulation of biological process. In contrast, only 1 cellular component, organelle envelope lumen, was significantly downregulated.

However, after mechanical stretching for 24 h, the number of significantly upregulated gene categories was much less than that of downregulated ones. The former only involved 1 molecular function, phosphate binding, and 1 biological process, multicellular organismal metabolic process. In contrast, the latter included 2 molecular functions, hyase activity and pattern binding, and 2 cellular components, extracellular region part and extracellular space. Moreover, 36 biological processes were also downregulated, including response to stimulus, multicellular organismal development, cell development, cell division, and anatomical structure formation involved in morphogenesis (Table S4).

Pathways Associated with Differentially Expressed Genes

KEGG pathway analysis was used to further analyze differentially expressed genes (p<0.05, more than twofold change) after mechanical stretching of Müller cells at both 1 and 24 h. The enrichment analysis revealed that 16 pathways were significant in differentially expressed genes at 1 h, and 20 pathways were significant at 24 h (p<0.05). At 1 h, the top five pathways were ErbB signaling pathway, MAPK signaling pathway, Jak-STAT signaling pathway, pathways in cancer, and Wnt signaling pathway (Table 3). At 24 h, the top five pathways were MAPK signaling pathway and 4 metabolism pathways (Table 4).

The Protein-Protein Interactions analysis was further performed to identify the direct interaction of these genes products using GeneSpring GX 11.5 (Figure 3). The diagram illustrates particularly strong interaction centers for IL 6, Hbegf, Ptg2, and Myc at 1 h and for Fos at 24 h. In addition, all genes in the 1 h interaction network were upregulated whereas those in the 24 h network were downregulated.

Validation of Selected Differentially Expressed Genes by qPCR

Based on a combination of statistical analysis of microarray data and potential biological importance of the genes of interest, 16 genes were chosen for qPCR confirmation (Table 5). These 16 genes are related to response to stimulus (Arcg, Egr2, Jun, PVR, Myc, Dusp1, IL6, Ptg2, Adm, and Fos), cell proliferation (Arcg, Jun, Myc, IL6, Ptg2, Hbegf, and Atf3), tissue remodeling (Arcg, PVR, IL6, Loxd2, and Has2), and vasculogenesis (Epha2, Nrnl). Though genes varied slightly in the changes they showed between microarray and qPCR results, there was a clear consistency between the two techniques (Tables 6 and 7), validating the results obtained from microarray analysis.

Discussion

Herein, we first report the differential gene expression profile of Müller cells responding to cyclic mechanical stretching for 1 and 24 h. We identified a number of genes related with response to stimulus, cell proliferation, tissue remodeling, and vasculogenesis and also highlighted some pathways such as MAPK pathway that were significantly involved and that might account for mechanisms of the effects of mechanical forces on Müller cells.

In this study, we used the Flexcell vacuum-driven system to stretch Müller cells by subjecting flexible-bottom culture dishes to distension [8], which has become a standard model for studying the effects of mechanical forces on a variety of ocular cell types, including trabecular meshwork cells [9], retinal microvascular endothelial cells [10], lamina cribrosa cells [11], scleral fibroblasts [12,13], and retinal pigment epithelial cells [14]. Although there exist some differences in the stretching regimens employed in these studies, elongation of 15% [9,11,12] and cyclic stretching [9,11] was a popular regimen, and axial length elongation by 15% can be observed almost only in cases of pathological myopia. This is why we selected this regimen in this study.

Microarray data analysis identified more significantly differentially expressed genes under mechanical stretching in Müller cells at 24 h than at 1 h. However, when comparing the differentially expressed genes between these two time points, we were surprised to find that there were no overlapped differential genes except for Nrl and Egr2. Furthermore, these two genes were upregulated at 1 h but downregulated at 24 h. Another interesting finding was that at 1 h, 86% of differential genes were upregulated, whereas at 24 h, 74% of differential genes were downregulated. These data revealed that gene expression induced in Müller cells by mechanical stretching at an early time (1 h) was temporary, suggesting a possible distinct response pattern to mechanical stretching at different stages.

Gene ontology analysis showed that numerous biological processes were involved in Müller cells after stretching, suggesting the active response of Müller cells to mechanical strain. This confirmed the mecanoresponsivity of Müller cells, which is consistent with a previous report [7], though cellular reactions might vary widely under mechanical stretching in Müller cells in confluent cultures, compared to those in retinal tissues. Herein, we mainly focus on those genes that are related to cell proliferation, tissue remodeling, and vasculogenesis because these might participate in pathological processes of ocular diseases in which the retina is overstretched.
| Accession# | Gene | Gene Title | Fold* |
|------------|------|------------|-------|
| NM_017352 | Nr4a3 | Nuclear receptor subfamily 4, group A, member 3 | 15.14 |
| NM_133578 | Dusp5 | Dual specificity phosphatase 5 | 8.16 |
| NM_001008826 | LOC360231 | MHC class I RT1.O type 149 processed pseudogene | 7.29 |
| NM_012912 | Atf3 | Activating transcription factor 3 | 5.72 |
| NM_017123 | Areg | Amphiregulin | 5.47 |
| NM_138526 | Ccrn4l | CCR4 carbon catabolite repression 4-like | 5.43 |
| NM_017259 | Btg2 | BTG family, member 2 | 5.24 |
| NM_001108510 | Dusp8 | Dual specificity phosphatase 8 | 5.10 |
| NM_024388 | Nr4a1 | Nuclear receptor subfamily 4, group A, member 1 | 4.82 |
| XM_01056859 | Sprr1al | Small proline-rich protein 1A-like | 4.62 |
| NM_017259 | Btg2 | BTG family, member 2 | 4.43 |
| NM_021869 | Ereg | Epiregulin | 4.05 |
| NM_012945 | Hbegf | Heparin-binding EGF-like growth factor | 3.76 |
| NM_001014071 | Erfr1 | ERBB receptor feedback inhibitor 1 | 3.66 |
| NM_031707 | Homer1 | Homer homolog 1 (Drosophila) | 3.60 |
| NM_053883 | Dusp6 | Dual specificity phosphatase 6 | 3.53 |
| NM_012589 | IL6 | Interleukin 6 | 3.20 |
| NM_012603 | Myc | Myelocytomatosis oncogene | 3.11 |
| NM_053769 | Dusp1 | Dual specificity phosphatase 1 | 2.97 |
| NM_001079890 | Gprr5a | G protein-coupled receptor, family C, group 5, member A | 3.06 |
| NM_153724 | Rcan1 | Regulator of calcineurin 1 | 2.99 |
| NM_017180 | Phlda1 | Pleckstrin homology-like domain, family A, member 1 | 2.97 |
| NM_053633 | Egp2 | Early growth response 2 | 2.92 |
| NM_019238 | Nr4a2 | Nuclear receptor subfamily 4, group A, member 2 | 2.78 |
| NM_012953 | Fosl1 | Fos-like antigen 1 | 2.75 |
| NM_053713 | Klif4 | Kruppel-like factor 4 (gut) | 2.71 |
| NM_013058 | Id3 | Inhibitor of DNA binding 3 | 2.65 |
| NM_053382 | Tnfaip6 | Tumor necrosis factor alpha induced protein 6 | 2.56 |
| NM_001047858 | Srxn1 | Sulfiredoxin 1 homolog (S. cerevisiae) | 2.56 |
| NM_021835 | Jun | Jun oncogene | 2.35 |
| NM_031971 | Hspa1 | Heat shock 70kD protein 1 | 2.51 |
| NM_013153 | Has2 | Hyaluronan synthase 2 | 2.49 |
| NM_012620 | Serpine1 | Serine (or cysteine) peptidase inhibitor, clade E, member 1 | 2.48 |
| NM_001012046 | Spy2 | Sprouty homolog 2 | 2.43 |
| NM_031971 | Hspa1a | Heat shock 70kD protein 1A | 2.43 |
| NM_024381 | Gk | Glycerol kinase | 2.38 |
| NM_017232 | Ptgs2 | Prostaglandin-endoperoxide synthase 2 | 2.37 |
| XM_002788821 | LOC100360845 | Hypothetical protein LOC100360845 | 2.16 |
| NM_001108600 | Crem | cAMP responsive element modulator | 2.15 |
| NM_001014094 | Plectr2 | Phospholipid scramblase 2 | 2.14 |
| NM_019242 | Ifrd1 | Interferon-related developmental regulator 1 | 2.12 |
| NM_001169116 | RGD1306119 | Similar to transcriptional regulating protein 132 | 2.11 |
| NM_012620 | Serpine1 | Serine (or cysteine) peptidase inhibitor, clade E, member 1 | 2.09 |
| NM_023985 | Trib1 | Tribbles homolog 1 | 2.05 |
| NM_001047858 | Srxn1 | Sulfiredoxin 1 homolog | 2.05 |
| NM_017076 | PVR | Poliovirus receptor | 2.04 |
| NM_001108977 | Ephra2 | Eph receptor A2 | 2.02 |
| NM_001106779 | Nedd1 | Neural precursor cell expressed, developmentally down-regulated | 0.48 |
| XM_001077448 | Dact1 | Dapper, antagonist of beta-catenin, homolog 1 | 0.48 |
Table 1. Cont.

| Accession# | Gene          | Gene Title                      | Fold* |
|------------|---------------|---------------------------------|-------|
| NM_001107250 | Znf503        | Zinc finger protein 503         | 0.48  |
| NM_0809906  | Ddit4         | DNA-damage-inducible transcript 4 | 0.46  |
| NM_013148   | Htr5a         | 5-Hydroxytryptamine (serotonin) receptor 5A | 0.45  |
| XM_001072241 | Mamll2       | Mastermind like 2              | 0.40  |
| NM_001008767 | Tnip         | Thioredoxin interacting protein  | 0.34  |
| NM_001108654 | Tox          | Thymocyte selection-associated high mobility group box | 0.31  |

*Fold change greater than 1.0 represents increases, while less than 1.0 indicates decreases in stretching versus control group.

Indicates gene expression result obtained from microarray analysis was further verified using qPCR.

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The proliferation of Müller cells has been suggested to play a central role in the development of epiretinal membranes associated with proliferative vitreoretinopathy [5,15]. We identified two highly differentially expressed genes that were related to proliferation—Areg and Atf3. Areg, which is significantly upregulated at both 1 and 24 h, is a member of the epidermal growth factor family. Previous studies have shown that it could reactivate astrocytes and promote cell proliferation [16,17,18]. Atf3, which is significantly upregulated at only 1 h, is a member of the mammalian activation transcription factor/cAMP responsive element-binding protein family of transcription factors. It responded to cellular injury [19] and could enhance cell proliferation [20,21,22]. Thus, it can be inferred that Müller cells could sense the mechanical traction in proliferative vitreoretinopathy and were activated to proliferate, contributing to the development of epiretinal membranes.

In pathological myopia, during the progressive distension of the posterior pole, the retina, choroid, and sclera are subjected to constant mechanical force, as a result of which tissue remodeling occurs [1,2]. Active remodeling of the sclera in myopia has been intensively studied [23,24]. Scleral fibroblasts are responsive to mechanical strain [13], and they regulate extracellular matrix synthesis [12]. In comparison, retinal remodeling under mechanical stretching has attracted little attention. Herein, we identified some tissue remodeling related genes, for instance, PVR and Loxl2. PVR belongs to a transmembrane glycoprotein belonging to the immunoglobulin superfamily, and its expression could promote the production of matrix metalloproteinases-2 [25], a well-known regulator of tissue remodeling [26]. Loxl2, a member of the lysyl oxidase gene family, was also involved in matrix remodeling [27,28].

Mechanical force was also one postulated mechanism of myopic choroidal neovascularization during the progressive and excessive elongation of the anteroposterior axis [2]. Mechanical stretching induced the expression and secretion of angiogenic factors in retinal pigment epithelial cells [14]. Here, we show that mechanical stretching induces some other angiogenic factors in Müller cells, such as EphA2 and Nrn1. Soluble EphA2 receptor could inhibit retinal neovascularization [29], and it might become an effective target for ocular neovascularizations [30]. Nrn1, a neurotrophic factor, was recently identified to function as a novel angiogenic factor [31].

The mechanisms of mechanosening in Müller cells in response to stretching remain unclear. Using KEGG pathway analysis, we identified several significantly changed pathways at 1 and 24 h. Interestingly, only the MAPK pathway was involved at both time points. A previous study also reported that the MAPK pathway was activated in stretched Müller cells [7]. All these data highlight the MAPK pathway as a possible key pathway underlying the mechanosensitivity of Müller cells subjected to mechanical stretching. Moreover, this pathway has been intensively studied in stretched vascular cells [32,33,34]. Other pathways such as the TGF-beta signaling pathway and nitrogen metabolism were also triggered in stretched vascular cells [32,34].

The results of this study should be interpreted with caution because stretching during pathology is likely to be much slower than in our experiments. It is unknown whether the changes we found would occur during much slower stretching in vivo.

Conclusion In summary, this study identified several differentially expressed genes and related pathways in Müller cells subjected to mechanical stretching. These results indicate the complex mechanoresponsive nature of Müller cells, and they provide novel insights into possible molecular mechanisms that would account for many retinal diseases in which the retina is often subjected to mechanical forces, such as degenerative axial myopia and proliferative vitreoretinopathy.

Materials and Methods

Ethics Statement The animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols were reviewed and approved by the Animal Ethics Committee of Fudan University (Shanghai, China).

Primary Müller Cell Culture Müller cells were prepared from Sprague-Dawley rats on postnatal days 1–3. Briefly, isolated retinas were digested with 0.25% trypsin (Invitrogen, Carlsbad, CA), and dissociated retinal cells were then cultured at 37°C in 5% CO₂ and 95% air in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (Invitrogen, Carlsbad, CA) and 100 U/mL penicillin/100 μg/mL streptomycin. Confluent cultures were passaged no more than four times, and cultures with the same number of passages were used for each independent experiment [35]. Cells at passages 2 to 4 were used for experiments. Isolated cells were confirmed by positive staining of three Müller cell markers, antibodies glutamine synthetase [36], vimentin, and SOX9 (data not shown).
| Accession#  | Gene     | Gene Title                                      | Folda |
|------------|----------|-------------------------------------------------|-------|
| NM_001106637 | Gem      | GTP binding protein                             | 2.70  |
| XM_001063122 | LOC685277| Similar to liver-specific bHLH-Zip transcription factor | 2.66  |
| NM_053346   | Nm1β     | Neuritin 1                                      | 2.60  |
| NM_001191721 | Rps6ka6  | Ribosomal protein 56 kinase polypeptide 6       | 2.60  |
| NM_001109344 | RGD1562846| Similar to Docking protein 5 (Downstream of tyrosine kinase 5) | 2.55  |
| NM_019176   | Stmn4    | Stathmin-like 4                                 | 2.40  |
| NM_053802   | Tgfbi    | Transforming growth factor, beta induced        | 2.33  |
| NM_001014193| RGD1359529| Similar to chromosome 1 open reading frame 63   | 2.28  |
| NM_001107464 | Dact2    | Dapper, antagonist of beta-catenin, homolog 2    | 2.28  |
| NM_001167840 | IL1rap   | Interleukin 1 receptor accessory protein        | 2.27  |
| NM_001134986 | Rnfl80   | Ring finger protein 180                          | 2.25  |
| NM_031522   | Neu1     | Sialidase 1 (lysosomal sialidase)                | 2.20  |
| NM_130812   | Cdkn2b   | Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) | 2.19  |
| NM_001106909| RGD1309095| Similar to hypothetical protein BC015148        | 2.15  |
| NM_017094   | Ghr      | Growth hormone receptor                         | 2.06  |
| NM_001106047 | Loxl2β   | Lysyl oxidase-like 2                            | 2.05  |
| NM_001106550 | Nkain4   | Na+/K+ transporting ATPase interacting 4        | 0.50  |
| NM_001106134 | Ska1     | Spindle and kinetochore associated complex subunit 1 | 0.50  |
| NM_019189   | Hapln1   | Hyaluronan and proteoglycan link protein 1      | 0.49  |
| NM_175578   | Rcan2    | Regulator of calcineurin 2                      | 0.49  |
| NM_001107956| Car9     | Carbonic anhydrase 9                            | 0.49  |
| NM_019212   | Acta1    | Actin, alpha 1                                  | 0.49  |
| NM_022183   | Top2a    | Topoisomerase (DNA) II alpha                    | 0.49  |
| NM_138502   | MglI     | Monoglyceride lipase                            | 0.48  |
| NM_001106623| RGD1311164| Similar to DNA segment, Chr 6                   | 0.48  |
| NM_001039549| Ugt1a    | UDP glucuronosyltransferase 1 family, polypeptide A | 0.48  |
| NM_138905   | Ppap2b   | Phosphatidic acid phosphatase type 2B           | 0.48  |
| NM_031582   | Aoc3     | Amine oxidase, copper containing 3 (vascular adhesion protein 1) | 0.48  |
| NM_012545   | Ddc      | Dopa decarboxylase (aromatic L-amino acid decarboxylase) | 0.47  |
| NM_001106465| Ntng1    | Netrin G1                                       | 0.47  |
| NM_012715   | Admβ     | Adrenomedullin                                  | 0.46  |
| NM_00103222 | Rnd1     | Rho family GTPase 1                             | 0.46  |
| NM_001106306| Cpxm2    | Carboxypeptidase X (M14 family), member 2       | 0.45  |
| NM_001007648| Cdc3α    | Cell division cycle associated 3               | 0.45  |
| NM_181635   | Kif15    | Kinesin family member 15                        | 0.45  |
| NM_012550   | Ednra    | Endothelin receptor type A                      | 0.44  |
| NM_001108009| Rasgrp3  | RAS guanyl releasing protein 3 (calcium and DAG-regulated) | 0.44  |
| NM_022183   | Top2a    | Topoisomerase (DNA) II alpha                    | 0.44  |
| NM_024388   | Nr4a1    | Nuclear receptor subfamily 4, group A, member 1  | 0.44  |
| NM_172033   | Plekhh1  | Pleckstrin homology domain containing, family B (evectins) member 1 | 0.43  |
| NM_053848   | Opcml    | Opioid binding protein/cell adhesion molecule-like | 0.43  |
| NM_138905   | Ppap2b   | Phosphatidic acid phosphatase type 2B           | 0.43  |
| XM_001078892| Gbp4     | Guanylate binding protein 4                     | 0.42  |
| NM_181087   | Cyp26b1  | Cytochrome P450, family 26, subfamily b, polypeptide 1 | 0.42  |
| XM_001069190| RGD1563437| Similar to KIAA1217                            | 0.41  |
| NM_001011893| 4-Sep    | Septin 4                                        | 0.41  |
| NM_031834   | Sul1a1   | Sulfolipid transferase family, cytosolic, 1A, phenol-prefering, member 1 | 0.41  |
| XM_001059692| RGD1307396| Similar to RIKEN cDNA 633040615                 | 0.40  |
| NM_053633   | Egr2β    | Early growth response 2                         | 0.40  |
Table 2. Cont.

| Accession# | Gene     | Gene Title                              | Fold* |
|------------|----------|-----------------------------------------|-------|
| XM_001056542 | LOC679475 | Hypothetical protein LOC679475          | 0.39  |
| NM_017226   | Padi2    | Peptidyl arginine deiminase, type II    | 0.39  |
| NM_153737   | Sostdc1  | Sclerostin domain containing 1          | 0.39  |
| NM_013122   | Igfbp2   | Insulin-like growth factor binding protein 2 | 0.36  |
| NM_001107221 | C1qtnf7  | C1q and tumor necrosis factor related protein 7 | 0.34  |
| NM_022707   | Pln      | Phospholamban                           | 0.34  |
| NM_022197   | Fos*     | FBJ osteosarcoma oncogene               | 0.33  |
| NM_019292   | Car3     | Carboxic anhydrase 3                    | 0.32  |
| NM_02257    | Masp1    | Mannan-binding lectin serine peptidase 1| 0.31  |
| NM_031739   | Kcnj5    | Potassium voltage-gated channel, Shal-related subfamily, member 3 | 0.31  |
| NM_012598   | Lpl      | Lipoprotein lipase                      | 0.30  |
| NM_021576   | Nt5e     | 5’-Nucleotidase, ecto                   | 0.22  |
| NM_001135855 | Scara5   | Scavenger receptor class A, member 5 (putative) | 0.21  |

*Fold change greater than 1.0 represents increases, while less than 1.0 indicates decreases in stretching versus control group.

# Indicates gene expression result obtained from microarray analysis was further verified using qPCR.

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![Hierarchical cluster analysis of differentially expressed genes](image)

**Figure 1.** Hierarchical cluster analysis of differentially expressed genes (p<0.05, more than twofold change) at 1 and 24 h. Each row represents a probe and each column represents one sample. The values represent the fold changes compared with the corresponding control. Positive and negative fold changes are shown in red and green, respectively, as shown in the color bar. S, stretching group; C, control group.

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Application of Mechanical Stretching

To apply mechanical strain, the Müller cells were plated on type-I collagen-coated flexible silicone bottom plates (Flexcell International, USA) at an initial density of $2 \times 10^5$ cells per well (9.32 cm²). After seeding for 24 h, the cells were pretreated with serum-free DMEM for 24 h prior to the experiments to arrest their growth and to synchronize them. The Müller cells were then subjected to 15% cyclic stretching (strain magnitudes, 15%; frequency, 1 Hz; duration, 1 h and 24 h) that was produced by a computer-controlled vacuum (FX-4000T Strain Unit, Flexcell International) as previously described [37]. Briefly, the silicone bottom plates with cultured cells were placed on a vacuum manifold situated in an incubator. When a vacuum was applied to the bottoms of plates, controlled by a computer, the silicone membranes were deformed to a prearranged elongation percentage and returned to their original conformation once the vacuum was released. During this course, Müller cells were tightly attached to the silicone membrane surface, and the deformation of the membrane is directly transmitted to the cells. Müller cells cultured under the same conditions but with no applied mechanical strain were considered as the unstretched control.

RNA Isolation

At each time point (1 and 24 h), three totally independent experiments (3 stretched samples and 3 control samples) were conducted. After being mechanically strained, as described above, Müller cells were extensively washed with cold PBS and total RNA was extracted using TRIZOL Reagent (Life Technologies, Carlsbad, CA, US) following the manufacturer’s instructions.

| Table 3. Significantly changed pathways in Müller cells after stretching for 1 h. |
|----------------------------------|-----|-----------------|
| Pathway Name                     | Genes (n) | P-value         |
| ErbB signaling pathway           | 5      | 0.0000          |
| MAPK signaling pathway           | 9      | 0.0000          |
| Jak-STAT signaling pathway       | 3      | 0.0013          |
| Pathways in cancer               | 4      | 0.0013          |
| Wnt signaling pathway            | 3      | 0.0013          |
| Prion diseases                   | 2      | 0.0014          |
| Leishmania infection             | 2      | 0.0042          |
| Colorectal cancer                | 2      | 0.0051          |
| TGF-beta signaling pathway       | 2      | 0.0067          |
| Small cell lung cancer           | 2      | 0.0083          |
| Toll-like receptor signaling path| 2      | 0.0088          |
| GnRH signaling pathway           | 2      | 0.009           |
| Antigen processing and presentation | 2  | 0.0104          |
| Spliceosome                      | 2      | 0.0154          |
| Thyroid cancer                   | 1      | 0.0441          |
| Endocytosis                      | 2      | 0.0458          |
| **doi**:10.1371/journal.pone.0063467.t003** |

| Table 4. Significantly changed pathways in Müller cells after stretching for 24 h. |
|----------------------------------|-----|-----------------|
| Pathway Name                     | Genes (n) | P-value         |
| Glycerolipid metabolism          | 3      | 0.0001          |
| Phenylalanine metabolism         | 2      | 0.0004          |
| Nitrogen metabolism              | 2      | 0.0008          |
| MAPK signaling pathway           | 4      | 0.0013          |
| Tyrosine metabolism              | 2      | 0.0016          |
| Sphingolipid metabolism          | 2      | 0.0026          |
| Retinol metabolism               | 2      | 0.0066          |
| B cell receptor signaling pathway | 2      | 0.0089          |
| Metabolic pathways               | 6      | 0.0171          |
| Sulfur metabolism                | 1      | 0.0203          |
| Vascular smooth muscle contraction| 2      | 0.0209          |
| Axon guidance                     | 2      | 0.0227          |
| Other glycan degradation          | 1      | 0.0286          |
| beta-Alanine metabolism           | 1      | 0.0385          |
| Nicotinate and nicotinamide metabolism | 1  | 0.0401          |
| Pentose and glucuronate interconversions | 1  | 0.0401          |
| Histidine metabolism             | 1      | 0.0418          |
| Calcium signaling pathway         | 2      | 0.0431          |
| Ascorbate and aldarate metabolism | 1      | 0.0434          |
| Homologous recombination          | 1      | 0.0450          |
| **doi**:10.1371/journal.pone.0063467.t004** |
and RNA quality was confirmed using the Agilent 2100 Bioanalyzer. The qualified total RNA was further purified by RNase micro kit (QIAGEN, GmBH, Germany) and RNase-Free DNase Set (QIAGEN, GmBH, Germany).

**Microarray Hybridization and Analysis**

The total RNA was amplified, labeled, and purified by using GeneChip 3’IVT Express Kit (Affymetrix, Santa Clara, CA, US) following the manufacturer’s instructions to obtain biotin-labeled cRNA. Array hybridization and washing was performed using Affymetrix Rat Genome 230 2.0 Array Hybridization, Wash, and Stain Kit (Affymetrix, Santa Clara, CA, US) in Hybridization Oven 645 (Affymetrix, Santa Clara, CA, US) and Fluidics Station 450 (Affymetrix, Santa Clara, CA, US) following the manufacturer’s instructions. The slides were scanned using GeneChip® Scanner 3000 (Affymetrix, Santa Clara, CA, US) and Command Console Software 3.1 (Affymetrix, Santa Clara, CA, US) with default settings. Before the statistical analyses, all microarrays were subjected to quality and filtering criteria. All microarray data have been deposited in the NIH/NLM Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/projects/geo) provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) and are accessible through GEO Series accession number GSE 43516. The raw data were normalized using MAS 5.0 algorithm, Gene Spring Software 11.5 (Agilent Technologies, Santa Clara, CA, US).

Data analysis was carried out by using GeneSpring GX 11.5 Software. It was also used for performing gene hierarchical clustering. Student’s t-test (GeneSpring GX11.5) was used to identify genes that were differentially expressed between the stretched and the control groups at the level of significance \( p < 0.05 \), but we mainly focused on those genes that showed more than a twofold change in expression. Gene Ontology analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were
used to select, annotate, and visualize these genes by function and pathway.

**Real-Time PCR**

RNA extracted for the microarray experiments was used to generate cDNA for qPCR using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. The qPCR was sequenced using the ABI 7900 HT Sequence Detection System (Applied Biosystems). The reactions were set up with 5 μL SYBR Green PCR Master Mix (Takara, Shuzo, Kyoto, Japan), 0.4 μL 0.5 μM primer mixture, and 5 ng cDNA template. Real-time PCR was performed under the following conditions: 50°C for 120 s, 95°C for 15 s followed by 40 cycles at 95°C for 15 s, and 60°C for 60 s. The relative mRNA levels of the target genes were normalized to GAPDH. The sequences of the primers used for the amplifications (Shenggong Company, China) are shown in Table 5. Statistical analysis was performed using Student’s t-test (Stata, ver. 10.0; Stata Corporation, College Station, TX, USA), and a value of p < 0.05 was considered significant.

### Table 5. Primers Used for qPCR.

| Gene Symbol | Genebank | Primer Sequence | Product Size (bp) |
|-------------|----------|-----------------|-------------------|
| Areg        | NM_017123| F: CGCACCAATGGCATCATTA R: TTCTGCTTCTCTATTCTCGAA | 71 |
| Eg2         | NM_053633| F: TTCTCTGCCGCTTGTGATG R: ACACGGAGGATTTTTTTTTC | 89 |
| Jun         | NM_021835| F: GATGGCTTGTAGTGGCTCCGCTA R: ATCCGAATCTAGCTGTACCTC | 89 |
| PVR         | NM_017076| F: CCAGTGTTGGCGGACAGACGAGACGATGA | 68 |
| Atf3        | NM_012912| F: TCCCTCCACCAAAAAACCA R: CCACGGCATCCACCTCATCTGAGATG | 81 |
| Myc         | NM_012603| F: GCCCTGAGTTAGTGAAGAGA R: CCGAGTAGACCTCATAGTGACCTTT | 72 |
| Dusp1       | NM_053769| F: TCAGGCGCCTGGAAGAAGA R: CCGAGTAGACCTCATAGTGACCTTT | 72 |

Table 6. Validation of Microarray Gene Expression at 1 h by qPCR.

| Gene | Fold change (Mean±SD) | Microarray (n = 3) | RT-qPCR (n = 3) |
|------|-----------------------|--------------------|-----------------|
| Areg | 5.47 ± 2.50*          | 5.51 ± 2.46*       |                 |
| Atf3 | 5.72 ± 1.37*          | 6.94 ± 1.91*       |                 |
| IL6  | 3.20 ± 1.36*          | 3.38 ± 2.27*       |                 |
| Hbegf| 3.76 ± 0.73*          | 4.14 ± 1.95*       |                 |
| Eg2  | 2.92 ± 0.74*          | 3.02 ± 1.13*       |                 |
| Myc  | 3.11 ± 0.40*          | 2.96 ± 0.49*       |                 |
| Dusp1| 2.97 ± 0.53*          | 4.08 ± 1.54*       |                 |
| Has2 | 2.49 ± 0.76*          | 2.56 ± 1.23*       |                 |
| PtgS2| 2.37 ± 0.63*          | 4.91 ± 1.29*       |                 |
| Jun  | 2.35 ± 0.48*          | 2.40 ± 0.08*       |                 |
| PVR  | 2.04 ± 0.49*          | 2.01 ± 0.39*       |                 |
| Epha2| 2.02 ± 0.41*          | 1.90 ± 0.13*       |                 |

Table 7. Validation of Microarray Gene Expression at 24 h by qPCR.

| Gene | Fold change (Mean±SD) | Microarray (n = 3) | RT-qPCR (n = 3) |
|------|-----------------------|--------------------|-----------------|
| Nrn1 | 2.60 ± 1.02*          | 3.07 ± 0.93*       |                 |
| Areg | 1.89 ± 0.29*          | 2.56 ± 0.61*       |                 |
| Lox2 | 2.05 ± 0.14*          | 2.57 ± 1.37*       |                 |
| Jun  | 1.25 ± 0.20*          | 1.56 ± 0.27*       |                 |
| PVR  | 1.18 ± 0.10*          | 1.44 ± 0.31*       |                 |
| Adm  | 0.46 ± 0.18*          | 0.61 ± 0.41        |                 |
| Eg2  | 0.40 ± 0.13*          | 0.46 ± 0.18*       |                 |
| Fos  | 0.33 ± 0.08*          | 0.35 ± 0.06*       |                 |

*Genes validated at both 1 and 24 h.

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Supporting Information

Table S1 Up- and downregulated genes in Müller cells after stretching for 1 h.

Table S2 Up- and downregulated Genes in Müller cells after stretching for 24 h.

Table S3 Significantly Upregulated Gene Categories after stretching for 1 h.

References

1. Morgan J, Ohno-Matsui K, Saw S (2012) Myopia. Lancet 379: 1739–1748.
2. Neelam K, Cheung CM, Ohno-Matsui K, Lau TY, Wong TY (2012) Choroidal neovascularization in pathological myopia. Prog Retin Eye Res 31: 495–525.
3. Scebba J (2004) Anomalous posterior vitreous detachment: a unifying concept in vitreoretinal disease. Graefes Arch Clin Exp Ophthalmol 242: 690–698.
4. Wurm A, Pannicke T, Iandiev I, Francke M, Hollborn M, et al. (2011) Purinergic signaling involved in Müller cell function in the mammalian retina. Prog Retin Eye Res 30: 324–342.
5. Bringmann A, Iandiev I, Pannicke T, Wurm A, Hollborn M, et al. (2009) Cellular signaling and factors involved in Müller cell gliosis: neuroprotective and detrimental effects. Prog Retin Eye Res 28: 423–451.
6. Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, et al. (2006) Müller cells in the healthy and diseased retina. Prog Retin Eye Res 25: 397–424.
7. Lindqvist N, Liu Q, Zajdaciak J, Franze K, Reichenbach A (2010) Retinal glial (Müller) cells: sensing and responding to tissue stretch. Invest Ophthalmol Vis Sci 51: 1683–1690.
8. Brown TD (2000) Techniques for mechanical stimulation of cells in vitro: a review. Journal of Biomechanics 33: 3–14.
9. Luna C, Li G, Liton PB, Epstein DL, Gonzalez P (2009) Alterations in gene expression induced by cyclic mechanical stress in trabecular meshwork cells. Mol Vis 15: 334–344.
10. Suzuma I, Hata Y, Clermont A, Pokras F, Rook SL, et al. (2001) Cyclic stretch and hypertension induce retinal expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2: potential mechanisms for exacerbation of diabetic retinopathy by hypertension. Diabetes 50: 444–454.
11. Kirwan RP, Crean JK, Fenerty CH, Clark AF, O’Brien CJ (2004) Effect of cyclical mechanical stretch and exogenous transforming growth factor-beta1 on matrix metalloproteinase-2 activity in lamina cribrosa cells from the human optic nerve head. J Glaucoma 13: 327–334.
12. Shelton L, Rada JS (2007) Effects of cyclic mechanical stretch on extracellular matrix synthesis by human scleral fibroblasts. Exp Eye Res 84: 314–322.
13. Cui W, Bryant MR, Sweet PM, McDonnell PJ (2004) Changes in gene expression in response to mechanical strain in human scleral fibroblasts. Exp Eye Res 83: 275–284.
14. Seko Y, Seko Y, Fujikura H, Pang J, Tokoro T, et al. (1999) Induction of astrocytes after neural injury. J Neurosci 26: 7532–7540.
15. Sethi CS, Lewis GP, Fisher SK, Leitner WP, Mann DL, et al. (2005) Glial activation: an upstream signal for transition of quiescent astrocytes into reactive astrocytes after neural injury. J Neurosci 25: 7532–7540.
16. Liu B, Chen H, Johns TG, Neufeld AH (2006) Epidermal growth factor receptor activation: an upstream signal for transition of quiescent astrocytes into reactive astrocytes after neural injury. J Neurosci 26: 7532–7540.
17. Guo Y, Johnson EC, Cepurna WO, Dyck JA, Doser T, et al. (2011) Early gene expression changes in the retinal ganglion cell layer of a rat glaucoma model. Invest Ophthalmol Vis Sci 52: 1460–1473.
18. Munoz-Erazo L, Natali R, Provins JM, Madigan MC, King NJ (2012) Microarray analysis of gene expression in West Nile virus-infected human retinal pigment epithelium. Mol Vis 18: 730–743.
19. Haygi K, Yasunaga J, Satou Y, Ohshima K, Matsuoka M (2011) ATF3, an HTLV-I bZIP factor binding protein, promotes proliferation of adult T-cell leukemia cells. Retrovirology 8: 19.
20. Tamura K, Hsu B, Adachi S, Guney I, Kawauchi J, et al. (2005) Stress response gene ATF3 is a target of c-myc in serum-induced cell proliferation. EMBO J 24: 2390–2401.
21. McBrien NA, Jobling AI, Gente A (2009) Biomechanics of the sclera in myopia: extracellular and cellular factors. Optom Vis Sci 86: E23–E30.
22. Rada JA, Shelton S, Norton TT (2006) The sclera and myopia. Exp Eye Res 82: 185–200.
23. Enloe BM, Jay DG (2011) Inhibition of Necl-5 (CD155/PVR) reduces glioblastoma dispersal and decreases MMP-2 expression and activity. J Neurosurg 102: 223–235.
24. Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 8: 223–233.
25. Seth S, Wordlinger RJ, Clark AF (2012) Focus on molecules: lysyl oxidase. Exp Eye Res 104: 97–98.
26. Barker HE, Chang J, Cox TR, Lang G, Bird D, et al. (2011) LOXL2-mediated matrix remodeling in metastasis and mammary gland involution. Cancer Res 71: 1361–1372.
27. Chen J, Hicks D, Bramley-Sieders D, Cheng N, McCollam GW, et al. (2006) Inhibition of retinal neovascularization by soluble EphA2 receptor. Exp Eye Res 82: 664–673.
28. Wang JI, Liu YL, Li Y, Dai WB, Guo ZM, et al. (2012) EphA2 targeted deoxouridine stealth liposomes as a therapy system for choroidal neovascularization in rats. Invest Ophthalmol Vis Sci 53: 7348–7357.
29. Han D, Qin B, Liu G, Liu T, Ji G, et al. (2011) Characterization of neutrin as a novel angiogenic factor. Biochem Biophys Res Commun 415: 608–612.
30. Shyu KG (2009) Cellular and molecular effects of mechanical stretch on vascular cells and cardiac myocytes. Clin Sci 116: 377–389.
31. Haga JH, Li YS, Chinen S (2007) Molecular basis of the effects of mechanical stretch on vascular smooth muscle cells. J Biomech 40: 947–960.
32. Kurpinski K, Park J, Thakar RG, Li S (2006) Regulation of vascular smooth muscle cells and mesenchymal stem cells by mechanical strain. Mol Cell Biomech 3: 21–24.
33. Ye X, Ren H, Zhang M, Sun Z, Jiang AC, et al. (2012) ERK1/2 signaling pathway in the release of VEGF from Müller cells in diabetes. Invest Ophthalmol Vis Sci 53: 3481–3489.
34. Herne KE, Norenburg MD (1977) Muller cell localization of glutamine synthetase in rat retina. Nature 268: 654–655.
35. Liu B, Qu MJ, Qin KR, Li H, Li ZK, et al. (2008) Role of cyclic strain frequency and hypertension induce retinal expression of vascular endothelial growth factor. Cell Cycle 7: 1561–1567.
36. Riepe RE, Norenburg MD (1977) Müller cell localization of glutamine synthetase in rat retina. Nature 268: 654–655.
37. Liu B, Qu MJ, Qin KR, Li H, Li ZK, et al. (2008) Role of cyclic strain frequency in regulating the alignment of vascular smooth muscle cells in vitro. Biochim Biophys Acta 1781: 1497–1507.

Table S4 Significantly downregulated Gene Categories after stretching for 24 h.

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Author Contributions

Conceived and designed the experiments: XW GX JF. Performed the experiments: XW MZ. Analyzed the data: XW JF. Contributed reagents/materials/analysis tools: XW MZ ZS. Wrote the paper: XW GX.