

Cyclic stretch-induced VEGF expression is mediated by TGF- β in retinal pigment epithelial cells

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Abstract

Background: Vitreomacular adhesion (VMA) has been theorised to be a prospective risk factor for anti-vascular endothelial growth factor (VEGF) therapy. The mechanisms underlying VMA are not fully understood. Therefore, we investigated whether exposure to cyclic stretch was associated with increasing VEGF and related signalling pathways in vitro.

Methods: A cyclic stretch model of adult retinal pigment epithelial cell line-19 (ARPE-19) was conducted using a Flexcell-5000 strain system at a regimen of 10% prolongation stretch (1/2 sine, 1Hz). We observed the effect of cyclic stretch on VEGF and transforming growth factor- β (TGF- β) by using western blot and real-time polymerase chain reaction (PCR) for 3 h, 6 h, 9 h and 12 h. Enzyme-linked immunosorbent assay was used to measure the secretion of VEGF in the supernatant. To quantitatively detect the angiogenesis capacity of VEGF in vitro, tube formation assay was performed. The effects of TGF- β receptor I inhibitor SB335242 on stretch-induced VEGF protein expression and secretion were evaluated by western blot and ELISA. Components of the p38 mitogen-activated protein kinase (MAPK) and c-Jun NH₂-terminal kinase (JNK) in the stretch group were analysed using western blot.

Results: We discovered that cultured ARPE-19 undergoing cyclic stretch significantly increased VEGF and TGF- β in a time-dependent manner and tube formation. Stretch-induced VEGF expression was inhibited by TGF- β receptor I inhibitor SB335242. In vitro, we also verified P38 MAPK and JNK expression. In western blot analysis, mechanical stretch evaluated P38 and JNK protein expression.

Conclusion: In this study, we first demonstrated that cyclic stretch induces the increasing VEGF through TGF- β in RPE cells. Mechanical stretch stimulates VEGF in a time-dependent manner and maintains angiogenesis ability. It is thus possible that RPE cells under mechanical stretch, such as VMA, may generate high levels of TGF- β . Sequentially, the overexpressed TGF- β stimulates VEGF production, triggering CNV events and aggravating wAMD.

Keywords: Mechanical cyclic stretch; vascular endothelial growth factor; retinal pigment epithelium; transforming growth factor-beta; mitogen-activated protein kinase; vitreomacular adhesion.

Introduction

Age-related macular degeneration (AMD) is the leading cause of central blindness in the elderly population of the western world[1]. The most severe vision loss occurs in patients with neovascular AMD, known as wAMD[2]. Currently, the major anti-vascular endothelial growth factor therapies (anti-VEGF) are ranibizumab, bevacizumab and aflibercept[3]. Although anti-VEGF therapy has greatly changed the paradigm of wAMD treatment, it remains a challenge for non-response patients[4]. Several risk factors exist for non-response anti-VEGF, including single-nucleotide polymorphisms (SNPs), complement factor H (CFH), VEGF-A genes[5], choroidal neovascularisation (CNV) lesion and initial reading ability[4]. Recently, vitreomacular adhesion (VMA) has been theorised to be a prospective risk factor for anti-VEGF therapy. This hypothesis has been inferred from several studies that reported a broad influence of anti-

VEGF therapy efficiency in eyes affected by VMA compared with age-matched controls[2, 6-10]. VMA was found to be located in the area of choroidal neovascularisation (CNV)[11, 12].

Although the mechanisms underlying the aggregation of these conditions by VMA are not well understood, VEGF has been strongly implicated as a primary mediator in anti-VEGF therapy efficiency. VEGF is the principal stimuli for raising angiogenesis in the improvement of CNV and other neovascularisation disorders[13]. In vivo, mechanical stretch can increase the expression of VEGF in human retinal pigment epithelium (RPE) cells. Wu et al.[14] studied stretch-induced VEGF expression upregulated in an increment of approximately 25% strain in adult retinal pigment epithelial cell line-19 (ARPE)-19 cells. Similarly, Seko et al.[15] found that in rat RPE cells, VEGF could rapidly increase loaded with 15% elongation.

The molecular mechanisms underlying stretch-induced VEGF expression have not been researched extensively. Transforming growth factor beta (TGF- β) mediates stretch-induced VEGF expression in pulmonary arterial smooth muscle cells[16], coronary[17] and cardiac myocytes[18]. TGF- β is a pleiotropic cytokine that binds to membrane receptors bearing serine/threonine kinase activity, namely TGF- β receptors type I and II[19]. Binding of the active form of TGF- β to TGF- β receptor type II initiates phosphorylation of TGF- β receptor type I, which is followed by the phosphorylation regulation of the Smad-dependent and Smad-independent pathways[20, 21]. To date, the most extensively studied isoforms of TGF- β have been isoforms 1[22] and 2[23]. The signalling pathways that act downstream of TGF- β include canonical (Smads) and noncanonical (e.g., c-Jun NH₂-terminal kinase [JNK]/p38 mitogen-activated protein kinase [MAPK], extracellular signal-regulated kinase-1/2 [ERK1/2], etc.) pathways[24]. In the eyes, TGF- β appears to be related to cell growth, differentiation, apoptosis and angiogenesis[25-30]. More importantly, TGF- β has been reported to stimulate strong VEGF expression through mitogen-activated protein kinases (MAPKs) in RPE cell culture[28].

In this study, we examined the mechanism of stretch-induced VEGF expression in RPE cells. This is the first study to demonstrate that stretch-induced VEGF expression is mediated by a TGF- β dependent pathway. Moreover, RPE cell exposure to cyclic stretch promoted P38 mitogen-activated protein kinase (MAPK) and JNK activation.

Methods

2.1 Culture of ARPE-19 cells and human umbilical vein endothelial cells (HUVECs)

The ARPE-19 cells were cultured as describe previously[14]. The normal ARPE-19 cells purchased from the American Type Culture Collection (ATCC) were cultured in Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F12 (1:1) (DMEM/F12, Gibco, Grand Island, NY) containing 10% foetal bovine serum (FBS, Gibco, Australia) and 1% penicillin-streptomycin (Gibco, Grand Island, NY) in a humidified incubator at 37°C at 5% CO₂. The medium was replaced every two or three days.

HUVECs were purchased from ATCC and were grown under the manufacturer's recommendation using Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY) supplemented with 10% foetal bovine serum (FBS, Gibco, Australia) and 1% penicillin-streptomycin (Gibco, Grand Island, NY). Cells of passages 2 through 7 were used for experiments. Cells were grown at 37°C in a humidified incubator with 5% CO₂.

2.2 Application of cyclic stretch

After removal of culture medium, ARPE-19 cells were harvested for seedlings on 6-well Flexcell culture plates coated with collagen type I (Flexcell International Corporation, Burlington, CA, USA). ARPE-19 cells were seeded in rubber membrane (Fig.1A). And then using DMEM with 1% FBS serum starved until reaching subconfluency. Subsequently, ARPE-19 cells in fresh 1% FBS media were subjected to a cyclic stretch regimen of 10% prolongation stretch (1/2 sine, 1Hz) using a Flexcell® FX-5000 Tension system (Flexcell International Corporation, Burlington, CA, USA). Vacuum drawn through the Loading post pulls the rubber membrane downward at 37°C in a 5% CO₂ (Fig.1B). Cells and supernatant were collected at different time points.

2.3 Quantitative real-time PCR

According to the manufacturer's instructions, total RNAs were extracted using Trizol (Invitrogen, USA). The purity and concentration of RNA were measured by the ultraviolet spectrometer at an optical density ratio of 260/280 (OD₂₆₀/OD₂₈₀). Then, total RNA was treated with RNase-Free DNase (Thermo, Pittsburgh, PA). First-strand cDNA was synthesised by reverse transcriptase from 5 µg total RNA with oligo-d (T) primers. Real-time PCR was performed by using an iCycler IQ real-time PCR detection system (Thermo, Pittsburgh, PA) to measure the fluorescence produced by SYBR Green I (Thermo, Pittsburgh, PA). The negative control was obtained by performing PCR without cDNA. The thermal cycling conditions were: 3 min at 95°C, followed by 40 cycles at 95°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. All PCR reaction products were verified by melting curve analysis. TGF-β mRNA is consistent with TGF-β1 mRNA and TGF-β2 mRNA. TGF-β and VEGFA mRNA expression levels were quantified by calculating the average value of triplicate reactions, normalised by the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are shown in Table 1.

2.4 Western blot

Antibodies used in this study are listed as follows: VEGF165 (1:1000, ab1316, Abcam), TGF-β 1+2 (1:1000, ab124894, Abcam), P38 MAPK (1:2000, 8690S, Cell Signalling Technology), JNK (1:2000, 9252S, Cell Signalling Technology), Smad2/3 (1:1000, ab207447, Abcam), ERK1/2 (1:1000, ab36991, Abcam) and GAPDH (1:5000, ab8245, Abcam)

The protein concentration of the supernatant was measured by a BCA Protein Assay Kit (CW BIO, CHINA). Equivalent amounts of total proteins (30 µg) were loaded on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel after transferring to polyvinylidene difluoride (PVDF)

membrane. Then, the membranes were incubated with primary antibody. The secondary antibody was goat anti-rabbit immunoglobulin G/horseradish peroxidase (IgG HRP)-conjugated (1:5,000) or goat anti-mouse IgG (HRP)-conjugated (1:5,000). All western blots were developed by HRP with the detection reagent Western Lightning Plus ECL (Merck, Waltham, MA). Band densities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). These data were normalised by GAPDH expression.

2.5 Enzyme-linked immunosorbent assay [31]

Samples were performed using the Quantikine Human VEGF ELISA Kit (R&D Systems, Minneapolis, MN). An aliquot of 200 μ l of conditioned medium was used per well. The levels of antigenic VEGF in the serial dilutions of ARPE-19 supernatants were quantitated by modification of a double ligand ELISA.

2.6 Tube formation assay

Tube formation assay was performed by the published protocol by Bai[32]. Aliquots of 100 μ l of Matrigel (Cat#356231, BD Sciences) were added to each well of the 48-well plates, followed by incubation at 37°C for 30 min. The HUVECs (5×10^4) were seeded to the top of solidified Matrigel with 150 μ l of conditioned medium for 3 h at 37°C. Before start of the assay, the HUVECs can be treated with calcein AM (ab141420, Abcam) to visualise the cells using fluorescence[33]. On five photographs in each group, fields of the Matrigel were randomly chosen under the microscope, and the length of the tubes was easier quantitated using Image J Pro (National Institutes of Health, Bethesda, MD). The experiments were repeated three times.

2.7 Statistical analysis

Data are expressed as mean \pm SEM. Significant differences between groups were ascertained by one-way analysis of variance (ANOVA) in combination with all pair wise multiple comparison procedures using Bonferroni test. In all cases, values of $p < 0.05$ were considered as statistically significant.

Results

3.1 Characterisation of stretch-induced VEGFA and TGF- β expression in ARPE-19 cells

ARPE-19 cells were subjected to an instance of 10% cyclic stretch for the durations indicated in Fig. 2A. Cyclic stretch maximally increased VEGFA mRNA expression 1.7-fold after 3 h and 6 h ($p=0.001$). Although VEGF mRNA levels gradually declined after that 6 h, stretch-induced VEGF mRNA significantly upregulated 1.3-fold after 9 h ($p=0.023$) and 1.2-fold after 12 h ($p=0.043$) compared with the control group. As shown in Fig. 2B, an increase in TGF- β mRNA expression was initially evident at 3 h, which peaked at 6 h when expression was 2.3 ± 0.5 -fold greater than in the control group ($p=0.005$).

To evaluate whether stretch-induced VEGF165 and TGF- β mRNA expression resulted in increased VEGF and TGF- β protein levels, western blot was used for analysis. As displayed in Fig. 3B, cyclic stretch

increased VEGF165 expression in a time-dependent manner. VEGF165 expression was increased 1.1±0.07-fold (p=0.03), 1.2±0.03-fold (p=0.000) and 1.3±0.02-fold (p=0.000) after 6 h, 9 h and 12 h of cyclic stretch, respectively. The level of TGF-β protein expression was highest 1.8 times control value at 3h (P<0.01), and then declining to 1.2 fold (P<0.05) at 12h (Fig. 3C).

The secretion of VEGFA at different time points was detected by ELISA. The line graph showed that the level of VEGFA increased with the extension of a cyclic stretch (Fig. 3D). The average value was 3 h (499.85±10.963 pg/mL), 6 h (870.185±32.153 pg/mL), 9 h (1226.908±60.715 pg/mL) and 12 h (1761.053±37.873 pg/mL), respectively. There was a significant difference between the stretch group and the non-stretch group at each time point (P<0.05).

3.2 Evaluation of stretch-induced TGF-β signalling pathways

The relative expression of JNK protein was significantly higher than that of the same time at 3 h (1.162±0.075-fold), 6 h (1.171±0.043-fold), 9 h (1.226±0.013-fold) and 12 h (1.1226±0.043-fold), respectively (p>0.05) (Fig. 4A-B). The relative expression levels of P38 MAPK protein in the stretch group at different time points were 3 h (1.214±0.056-fold), 6 h (1.312±0.044-fold), 9 h (1.216±0.05-fold) and 12 h (1.194±0.052-fold), which were significantly higher than those of the control group (P<0.01) (Fig. 4C).

3.3 Role of TGF-β in Stretch-induced VEGF Expression

The results in Figures 5A and 5B show that under the action of SB525334, the increase of VEGF165 expression induced by cyclic stretch was inhibited, which was significantly different from that of the non-inhibitor group (P<0.01); at the same time, there were no tension loading and blank control groups. In comparing (weakness without inhibitors), the relative protein expression of VEGF165 was also significantly reduced (P<0.05). With the prolongation of the action time, SB525334 exerted a continuously weakening effect on the traction force-induced VEGF secretion, and there was a statistically significant difference between the time points and the no inhibitor control group (P<0.01). To gain further insight into the potential expression, we detected the media by ELISA (Fig.5C). Treatment with SB525334 for a series time that could significantly inhibited VEGF expression (P<0.01). The average value was 3 h (0.16±0.028 fold), 6 h (0.17±0.013 fold), 9 h (0.2±0.016 fold) and 12 h (0.15±0.024 fold), respectively.

3.4 Stretch-induced VEGF stimulated HUVEC tube formation

Since the formation of tube-like structures is essential for angiogenesis[34], the ability of stretch-induced VEGF to promote tube formation was further investigated in Matrigel. Cultured HUVECs in vitro were incubated with the conditioned medium of the stretch group and control group for 3 h at 37°C in 5% CO₂. Typical tube-like structures were observed after fluorescent staining (Fig. 6A). The analysis by Image J software showed that the tube of length in the stretch group was significantly longer than that of the control group at 6 h (116±8%, P<0.05), 9 h (115±8%, P<0.05) and 12 h (127±4%, P<0.01), respectively (Fig. 6B).

Discussion

Our study describes that cyclic stretch strongly increases VEGF expression in a time-dependent manner and promotes angiogenesis in human RPE cells. Meanwhile, stretch-induced VEGF expression is predominantly dependent on TGF- β and upregulates P38 MAPK and JNK cytokines.

Currently, the gold standard treatment for CNV is an intravitreal injection of anti-VEGF drugs, albeit with limited visual recovery in wAMD combined with VMA[4, 35]. Gao et al. [35] suggested that VMA traction had a significant influence on the treatment of anti-VEGF. Thus, a deeper understanding of VMA traction aetio-pathogeny is warranted. Stemming from the application of Flexcell-5000 tension system to stimulate ARPE-19 cells, the expression and secretion of VEGF protein were significantly increased over time, while VEGF mRNA peaked at 6 h and then decreased slightly, indicating that mechanical stretch could regulate VEGF expression. The time course of VEGF expression in response to cyclic stretch in ARPE-19 was similar to that reported by Wu et al.[14], who also detected a sharp decrease after withdrawal of cyclic stretch. Seko et al.[15] found that VEGF expression was remarkably upregulated at 24 h in the 0.2 Hz 10% variability group, whereas it was increased at 1 h or 3 h in the 1 Hz 15% variability group in rat RPE cells. Compared with our results, these discrepancies may relate to the use of the older model device (Flexcell-3000 tension system) and the inconsistent mechanical parameters, or the use of rat cells rather than human cells.

In addition, we noticed that the higher concentration of VEGF measured by ELISA could significantly induce the differentiation of endothelial cells to tube-like cells, and length of the tube was dramatically longer than that of the control group. Since the formation of tube-like structures is essential for angiogenesis, these results indicate that cyclic stretch could induce high expression of VEGF and has the potential to promote angiogenesis. CNV is an important pathological process in a variety of chorioretinal diseases, and both angiogenesis and vasculogenesis are involved[36]. Our study demonstrated cyclic stretch-induced VEGF expression could promote angiogenesis, which may partly illustrate the reason why the presence of VMA influences anti-VEGF efficacy. However, the mechanism of stretch-induced VEGF expression is unknown.

In other systems, including pulmonary arterial smooth muscle cells[16], coronary[17], rat cardiac myocytes[37] and heart[38] induce stretch-induced VEGF expression through a TGF- β mediated pathway. Our data revealed that the expression of TGF- β mRNA and protein significantly increased. Meanwhile, the peak of appearance time preceded the peak of VEGF. Our study is the first cell experiment detecting the mechanisms of stretch-induced VEGF expression in RPE cells. The upregulation of TGF- β may significantly indicate that it could participate in stretch-induced VEGF expression. Previous studies have reported that TGF- β strongly enhanced VEGF mRNA transcription and protein expression[39-41]. We report here that TGF- β receptor I inhibitor SB525334 could prevent stretch-induced VEGF expression over time. These findings indicated that TGF- β may work as a major intermediate in stretch-induced VEGF upregulation. Besides, a series of studies has confirmed that VEGF is the central link in promoting angiogenesis in the development of CNV[13], suggesting that mechanical stretch is a critical trophic

factor for the expression of aberrant neovascularisation. In vivo, the TGF- β mRNA expression could be detected in retinal pigment epithelial cells, fibroblast-like cells and the endothelium of the neovascular region[39]. Also, TGF- β mediates collagen remodelling and scar contraction in the later stage of CNV, leading to scar tissue formation[42]. In an animal model, Nagineni et al.[28] showed that a high level of TGF- β is present in the retina of CNV patients, and Wang et al.[43] reported that the TGF- β /Smad pathway may function as an angiogenesis promoter in CNV development. These studies demonstrate that TGF- β plays a critical role in CNV development.

We further explored the potential pathway through which TGF- β was involved in stretch-induced VEGF expression. In this study, JNK and P38 MAPK were activated and shown significant time-dependence under cyclic stretch. In the previous studies, Xu et al.[44] used magnetite beads to apply strain force to RPE cells, and Eri et al.[45]⁴⁴ used mechanical scrapers to confirm that P38 MAPK activation can be induced by mechanical stretch, which agrees with our results. However, none of them found the activation of JNK under any mechanical force. Moreover, Gao et al.[46] used a 20% elongation stretch for stimulation for 6 h and 24 h and failed to change the JNK expression. We suspect that the above-mentioned differences may be related to multiple mechanical stimulation models and mechanical parameters. Much remains to be elucidated regarding this mechanism. Furthermore, there couldn't ignore with regards to the use of ARPE-19 cells, given the departure in the expression of important RPE-specific genes, in comparison to human RPE cells such as primary cell lines and freshly isolated RPE cells.

In summary, the results first demonstrated that cyclic stretch induces the expression of TGF- β that in turn increases VEGF expression in RPE cells. Mechanical stretch stimulates VEGF in a time-dependent way and maintains angiogenesis ability. Furthermore, P38 MAPK and JNK appear to be upregulated following cyclic stretch. It is thus possible that RPE cells under mechanical stretch, such as VMA, may produce high levels of TGF- β . Sequentially, the overexpressed TGF- β stimulates VEGF production, triggering CNV events and aggravating wAMD. Therefore, these cues could explain the molecular mechanisms of non-response anti-VEGF therapy in vivo, suggesting that the release of VMA traction or TGF- β inhibitors might provide an alternative to traditional methods in the clinical field.

Conclusions

Mechanical stretch could induce VEGF expression in a time-dependent manner; these changes is mediated by TGF- β in RPE cells.

Abbreviations

VMA: vitreomacular adhesion; VEGF: anti-vascular endothelial growth factor; ARPE-19: A spontaneously arising RPE cell line derived in 1986 by Amy Aotaki-Keen from the normal eyes of a 19-year-old male who died from head trauma in a motor vehicle accident; TGF- β : transforming growth factor- β ; PCR: real-time polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; MAPK: mitogen-activated protein kinase; JNK: c-Jun NH₂-terminal kinase; AMD: age-related macular degeneration; SNPs: single-

nucleotide polymorphisms; CNV: choroidal neovascularisation; CFH: complement factor H; FBS: Fetal bovine serum; DMEM-F12: Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F12; HUVECs: human umbilical vein endothelial cells; RPMI: Roswell Park Memorial Institute; ANOVA: One-way analysis of variance.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors declare that they have no conflict of interest with this submission.

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Authors' contributions

Involved in the design of the study (JQ.C.,W.L.); conduct of the study (JQ.C.,S.W., J.Z.,Q.L.); analysis of the data (JQ.C.S.W., W.L.); preparation of the manuscript (JQ.C.,S.W., J.Z.,Q.L., W.L.); and critical revision of the manuscript (JQ.C.,S.W., W.L.). All authors read and approved the final manuscript.

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Tables

Table 1 - Primer Sequences

Gene	Forward Primer	Reverse Primer
(GeneBank accession No.)		
VEGFA	TCTCTACCCCAGGTCAGACG	AGCAATGTCCTGAAGCTCCC
(NM_001025366.2)		
TGF-β1	CCGTGGGATACTGAGACACC	CTCGAGGGAAAGCTGAGGTC
(NM_000660.6)		
TGF-β2	CTTTGGATGCGGCCTATTGC	CTGCTGTGCTGAGTGTCTGA
(NM_003238.4)		
NM_001135599.3)		
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
(NM_002046)		

Figures

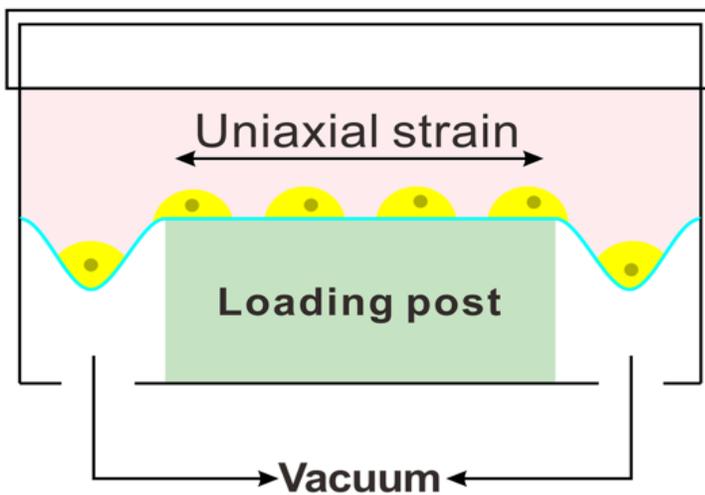
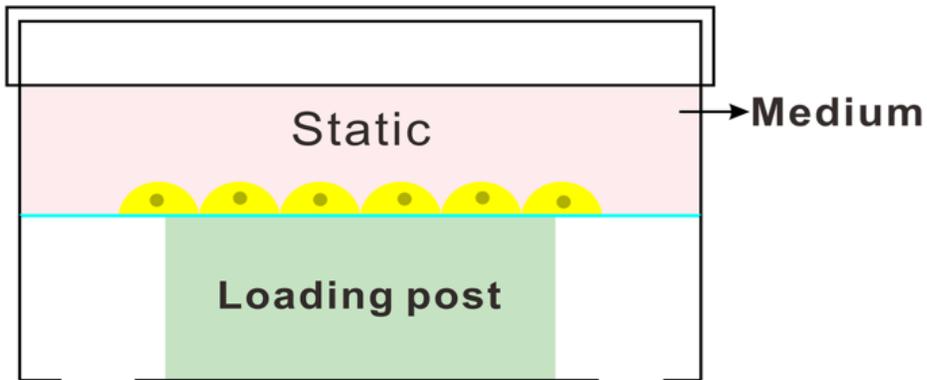


Figure 1

Schematic diagram of one well of a culture plate (A) shown from above, the rubber membrane coated with Collagen type I is drawn in non-stretch. Fig.1B showed that vacuum drawn through the Loading post pulls

the rubber membrane downward in a cyclic stretch regimen of 10% prolongation stretch (1/2 sine, 1Hz). Thereafter, cells and supernatant were collected at different time points.

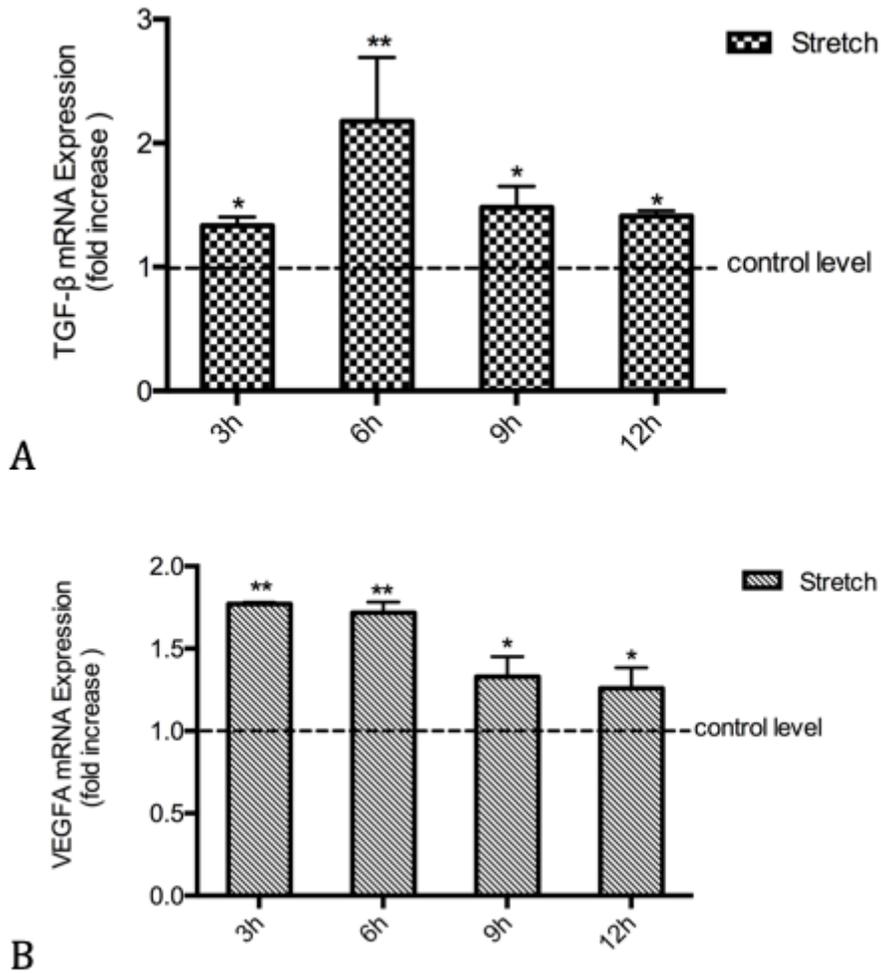


Figure 2

Cyclic stretch increases VEGFA mRNA and TGF-β mRNA expression VEGF mRNA (A) and TGF-β mRNA (B) expression showed a statistically significant time-dependent increase after normalization to the control group. The experiment was repeated three times with similar results. *P < 0.05, **P < 0.01.

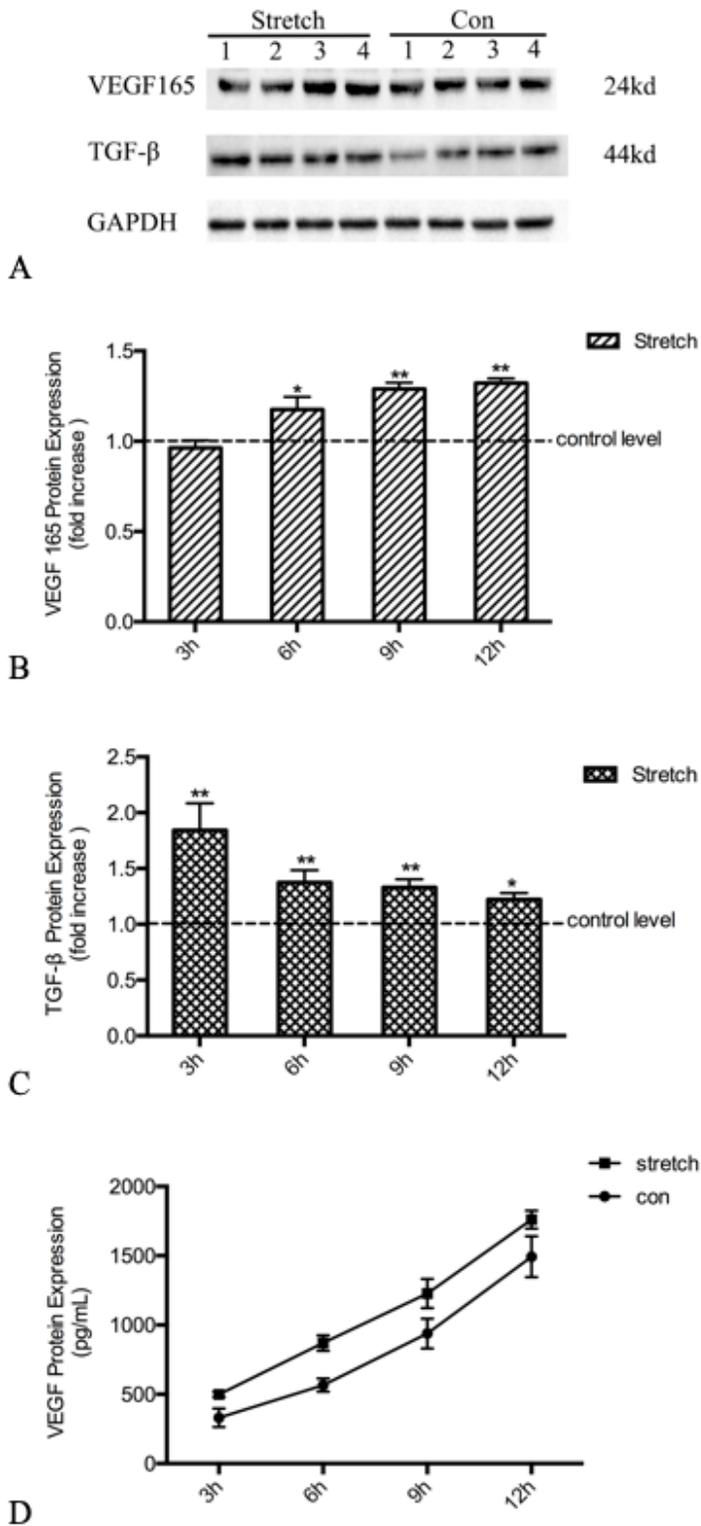


Figure 3

Effect of cyclic stretch on VEGF and TGF- β protein A Western blotting analysis of stretch induced VEGF165 protein and TGF- β protein undergo stretch and non-stretch group. The location of a 24kd and 44kd are respectively shown in figure. Results are expressed as relative VEGF165 protein (B) and TGF- β (C) protein compared with control levels. D The collected supernatant was analyzed by ELISA to examine the levels of secreted VEGFA. Data was from three independent experiments. *P < 0.05, **P < 0.01.

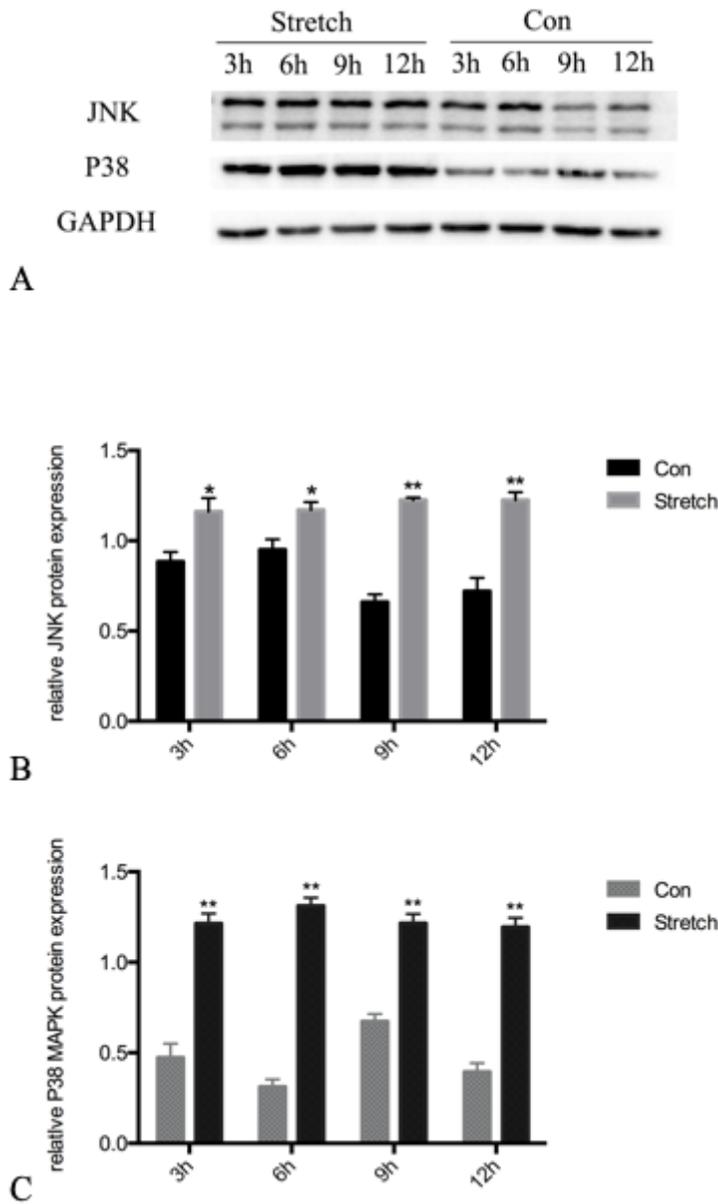


Figure 4

Cyclic stretch increases P38 MAPK and JNK expression A Western blotting analysis of stretch induced P38 MAPK, JNK, Smad2/3 and ERK1/2 in stretch and non-stretch group. As is shown, P38 MAPK and JNK are respectively significant increasing compared with control group. Smad2/3 and ERK1/2 were not increased in any samples of stretch group. B-C Quantitative analysis of P38 MAPK and JNK abundance. Data was from three independent experiments. *P < 0.05, **P < 0.01.

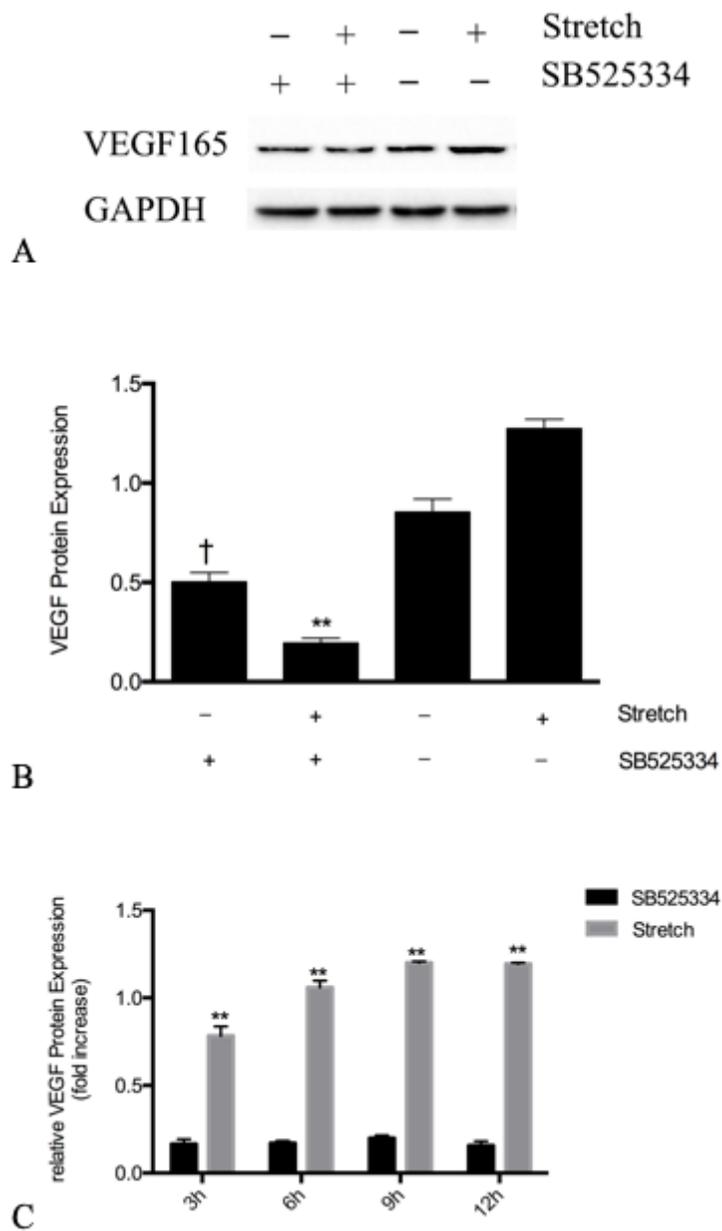
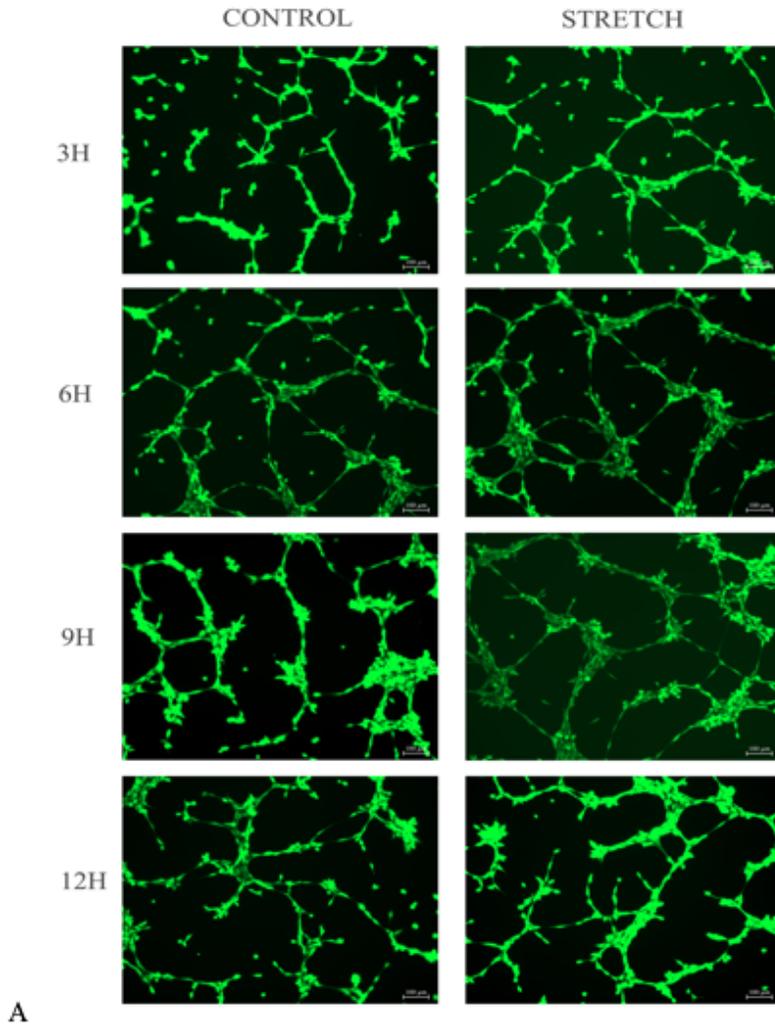
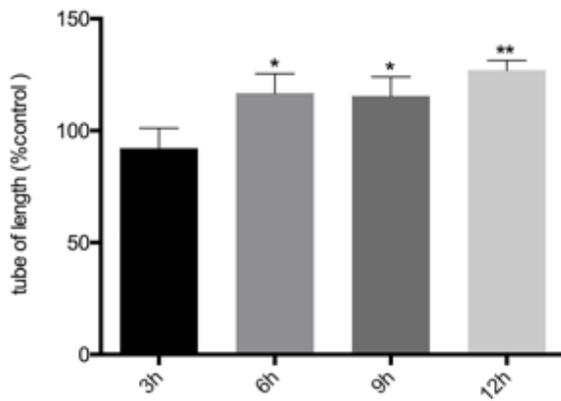


Figure 5

Cyclic stretch-induced VEGF expression is TGF- β dependent ARPE-19 was exposed to 10% elongation stretch for 3h in the present (+) or absence (-) of an antibody against TGF- β receptor I (SB525334, 20uM). A. Four groups' cell lysates was subjected to western blot using and polyclonal antibody against VEGF165. B. Band density Quantitation of multiple experiments is shown. C. Stretch and SB525334 were started at the same time, and condition media was evaluated by ELISA compared with non-stretch or SB525334 group after 3h, 6h, 9h and 12h. The fold change was calculating by dividing the control group. Data was from three independent experiments. *P < 0.05, **P < 0.01.



A



B

Figure 6

Fluorescence microscopy imaging and quantification of tube formation Typical tube formation was seen in any of the samples with conditioned medium (A). The length of tube-like formation was evaluated by image J software. Quantitative data are presented as mean \pm SEM (n = 3) in (B). *P < 0.05, **P < 0.01.