

# Osmotic induction of placental growth factor in retinal pigment epithelial cells in vitro: contribution of NFAT5 activity

Margrit Hollborn<sup>1</sup> · Konrad Reichmuth<sup>1</sup> · Philipp Prager<sup>1</sup> · Peter Wiedemann<sup>1</sup> · Andreas Bringmann<sup>1</sup> · Leon Kohen<sup>1,2</sup>

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**Abstract** One risk factor of neovascular age-related macular degeneration is systemic hypertension; hypertension is mainly caused by extracellular hyperosmolarity after consumption of dietary salt. In retinal pigment epithelial (RPE) cells, high extracellular osmolarity induces vascular endothelial growth factor (VEGF)-A (Hollborn et al. in *Mol Vis* 21:360–377, 2015). The aim of the present study was to determine whether extracellular hyperosmolarity and chemical hypoxia trigger the expression of further VEGF family members including placental growth factor (PIGF) in human RPE cells. Hyperosmotic media were made up by addition of 100 mM NaCl or sucrose. Chemical hypoxia was induced by CoCl<sub>2</sub>. Gene expression was quantified by real-time RT-PCR, and secretion of PIGF-2 was investigated with ELISA. Nuclear factor of activated T cell 5 (NFAT5) was depleted using siRNA. Extracellular hyperosmolarity triggered expression of VEGF-A, VEGF-D, and PIGF genes, and secretion of PIGF-2. Hypoosmolarity decreased PIGF gene expression. Hypoxia induced expression of VEGF-A, VEGF-B, VEGF-D, and PIGF genes. Extracellular hyperosmolarity and hypoxia produced additive PIGF gene expression. Both hyperosmolarity and hypoxia induced expression of KDR and FLT-4 receptor genes, while hyperosmolarity caused neuropilin-2 and hypoxia neuropilin-1 gene expression. The hyperosmotic, but not the

hypoxic, PIGF gene expression was in part mediated by NFAT5. The expression of PIGF in RPE cells depends on the extracellular osmolarity. The data suggest that high consumption of dietary salt may exacerbate the angiogenic response of RPE cells in the hypoxic retina via transcriptional activation of various VEGF family member genes.

**Keywords** Retinal pigment epithelium · Osmolarity · Hypoxia · PIGF · VEGF · NFAT5

## Introduction

Age-related macular degeneration (AMD) is the primary cause of blindness in elder individuals [1]. Key characteristics of wet (neovascular) AMD are choroidal neovascularization, i.e., the development of abnormal vessels which grow from the choriocapillaris towards the retinal pigment epithelium (RPE) and the subretinal space, and subretinal edema which results from leakage of newly formed vessels and opening of the outer blood-retinal barrier that is normally created by tight junctions between RPE cells [2]. Hypoxia of the outer retina is an important pathogenic condition of choroidal neovascularization [3]. Malfunction of the RPE and edema formation cause photoreceptor degeneration resulting in reduction of the visual acuity [4]. Vascular endothelial growth factor-A (VEGF-A) is the major angiogenic factor that promotes choroidal neovascularization and edema [5]. RPE cells are a source of angiogenic factors like VEGF-A in the retina [6]. However, it has been shown that the action of VEGF-A alone is insufficient to produce choroidal neovascularization [7]. The action of further angiogenic factors is mandatory for the angiogenic effect of VEGF-A [8, 9]. These findings has led to the suggestion that the simultaneous inhibition of various

Margrit Hollborn and Konrad Reichmuth have contributed equally to the work.

✉ Margrit Hollborn  
hollbm@medizin.uni-leipzig.de

<sup>1</sup> Department of Ophthalmology and Eye Hospital, Faculty of Medicine, University of Leipzig, Liebigstrasse 10-14, 04103 Leipzig, Germany

<sup>2</sup> Helios Klinikum Aue, Aue, Germany

angiogenic factors will have greater therapeutic benefit regarding antiangiogenesis in wet AMD than inhibition of VEGF-A alone [10]. In addition to VEGF-A, RPE cells produce further members of the human VEGF family, i.e., VEGF-B, -C, and -D, as well as placental growth factor (PlGF) [11–16].

It has been shown that, in the ischemic retina, the action of PlGF is indispensable for the angiogenic effect of VEGF [17]. PlGF promotes experimental choroidal neovascularization [18, 19]; the growth of choroidal vessels is inhibited in PlGF-deficient mice [20]. PlGF was shown to induce proliferation of vascular endothelial cells and vascular permeability via a mechanism that includes potentiation of the action of VEGF-A [21–23].

Various factors including age, race, genetic markers and lifestyle factors such as sun light exposure, cigarette smoking, and nutrition influence the risk of AMD. Furthermore, systemic hypertension is associated with a higher risk of wet AMD [24–26]. The primary condition which induces acute hypertension is a rise of the extracellular osmolarity after consumption of dietary salt [27]. There are age-dependent increases of the extracellular osmolarity and the hypertension-inducing effect of dietary salt [28, 29]. On the other hand, the use of antihypertensive medication was shown to be unassociated with the risk of early AMD and with an increased risk of wet AMD [24, 30]. Therefore, it was suggested that hypertension-related conditions such as high extracellular osmolarity and high extracellular salt (NaCl) rather than hypertension *per se* may aggravate neovascular AMD [31]. Indeed, it has been described that these conditions induce expression and secretion of VEGF-A in RPE cells [31]. However, it is unknown whether high extracellular osmolarity and NaCl, respectively, also induce expression of further VEGF family members like PlGF in RPE cells. Therefore, we determined the effects of high extracellular NaCl on the gene expression levels of VEGF family members in human RPE cells and found that this condition induces expression of VEGF-D and PlGF genes. We further examined the intra- and extracellular signaling mechanisms which contribute to the gene expression of PlGF in RPE cells under high NaCl- and hypoxic conditions, and investigated whether the activity of the osmosensitive nuclear factor of activated T cell 5 (NFAT5) [32] mediates the hyperosmotic expression of the PlGF gene in RPE cells.

## Materials and methods

### Materials

The culture components were obtained from Gibco BRL (Paisley, UK). Recombinant human transforming growth

factor (TGF)- $\beta$ 1 was obtained from R&D Systems (Abingdon, UK). AG1478, HIF inhibitor, LY294002, PD98059, SP600125, and SU1498 were purchased from Calbiochem (Bad Soden, Germany). SB203580 and caffeic acid phenethyl ester (CAPE) were obtained from Tocris (Ellisville, MO). Stattic was from Enzo Life Science (Plymouth Meeting, PA), and PD173034 was kindly provided by Pfizer (Karlsruhe, Germany). Small interfering ribonucleic acid (siRNA) against human NFAT5 and nontargeted siRNA were from Qiagen (Hilden, Germany; catalog numbers SI03076906 and 1027280) and Santa Cruz Biotechnology (Heidelberg, Germany; catalog numbers sc-43968 and sc-37007), respectively. AG1296, SB431542, and the other substances used were purchased from Sigma-Aldrich (Taufkirchen, Germany).

### Human material

The study was performed according to the Declaration of Helsinki for research involving human subjects; the use of human retinal cells was approved by the University of Leipzig Ethics Committee (approval #745, 07/25/2011). Eyes of post-mortem cornea donors without reported eye disease were obtained from 13 females and 11 males within 48 h of death; the written informed consent was obtained from the relatives. The age of the donors was 36–79 years (mean  $\pm$  SD,  $56.2 \pm 16.0$  years for females, and  $55.5 \pm 16.1$  years for males). The donors suffered from following end-stage diseases: multiple organ failure ( $n = 8$ ), subarachnoid hemorrhage ( $n = 4$ ), cerebral hemorrhage ( $n = 3$ ), cardiac infarction ( $n = 3$ ), pulmonary embolism ( $n = 2$ ), lung failure and hepatocerebral syndrome due to chronic liver cirrhosis ( $n = 1$ ), traumatic brain injury ( $n = 1$ ), cerebral edema ( $n = 1$ ), and heparin-induced thrombocytopenia, renal failure, and bronchopneumonia ( $n = 1$ ). Data received with cells of younger and aged donors, and with cells of females and males, were not significantly different (not shown).

### Cell culture

Preparation and culture of RPE cells was described previously [33]. Cell lines of different donors were used in passage numbers 3–5. When a confluency of approximately 90 % was achieved, the cells were cultured in serum-free medium for 16 h. Thereafter, test substances were added to the serum-free medium. Extracellular hyperosmolarity was induced by addition of NaCl (100 mM) or sucrose (100 mM) to the culture medium. Extracellular hypoosmolarity (60 % osmolarity) was achieved by addition of distilled water.  $\text{CoCl}_2$  (150  $\mu\text{M}$ ) was used to induce chemical hypoxia. Pharmacological inhibitors were preincubated for 30 min.

### Extraction of total RNA and cDNA synthesis

Extraction of the total RNA was performed with the InviTrap Spin Universal RNA Mini Kit (Stratec Molecular, Berlin, Germany). The  $A_{260}/A_{280}$  ratio of the optical density of RNA samples in agarose gel electrophoresis blots (measured with NanoDrop1000; peQLab, Erlangen, Germany) was 1.95–2.03, suggesting adequate RNA quality. The RNA samples were treated with DNase I (Roche, Mannheim, Germany), and cDNA was synthesized from 1  $\mu$ g RNA with a reverse transcription kit (Fermentas, St. Leon-Roth, Germany).

### Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Real-time RT-PCR was carried out using the Single-Color Real-Time PCR Detection System (BioRad, Munich, Germany). The primer sequences are given in Table 1. The amplification reaction mixture (15  $\mu$ l) consisted of 7.5  $\mu$ l of 2  $\times$  iQ SYBR Green Supermix (BioRad), specific primer set (0.2  $\mu$ M), and 1  $\mu$ l (1.25 ng) cDNA. Forty five amplification cycles were carried out for each sample (1 cycle of denaturation at 95  $^{\circ}$ C for 3 min, 45 cycles denaturation at 95  $^{\circ}$ C for 30 s, annealing at 58  $^{\circ}$ C for 20 s, extension at 72  $^{\circ}$ C for 45 s, and melting curve at 55  $^{\circ}$ C with the temperature gradually increased for 0.5  $^{\circ}$ C up to 95  $^{\circ}$ C). To prove the correct lengths of PCR products, the samples were analyzed with agarose gel electrophoresis. RT-PCR for  $\beta$ -actin messenger (m)RNA was used as an internal control. The results were analyzed with the  $2^{-\Delta\Delta C_t}$  method.

### Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded in 12-well plates ( $3 \times 10^3$  cells per well). After reaching a confluency of approximately 90 %, the medium was changed to serum-free medium for 16 h. Thereafter, NaCl (100 mM) or TGF- $\beta$ 1 (10 ng/ml) were added to the serum-free medium. After 24 h, the level of PIGF-2 protein in the cultured media (100  $\mu$ l) was quantified with ELISA (R&D Systems; catalog number DPG00).

### siRNA transfection

Cells were seeded in 12-well plates ( $7 \times 10^4$  cells per well). When a confluency of 60–80 % was achieved, NFAT5 siRNA or nontargeted siRNA were transfected into the cells with HiPerfect reagent (Qiagen) in fetal bovine serum (10 %)-containing F-10 medium. After 48 h, the cells were cultured 2 h in serum-free medium and then for

6 and 24 h in serum-free iso- or hyperosmotic medium (+100 mM NaCl), or isoosmotic medium that contained  $\text{CoCl}_2$  (150  $\mu$ M). After RNA extraction, the level of PIGF mRNA was evaluated by real-time RT-PCR.

### Statistics

Each experiment was carried out with a cell line derived from one donor. Each test involved at least three experiments using cell lines of different donors. Data are shown as mean  $\pm$  SEM. Statistical analysis was carried out with Prism (Graphpad Software, San Diego, CA). Comparisons between groups were performed with one-way ANOVA followed by Bonferroni's multiple comparison test and Mann–Whitney  $U$  test. A value of  $P < 0.05$  was considered statistically significant.

## Results

### Osmotic regulation of VEGF isotype and PIGF genes

It has been shown that elevation of the extracellular osmolarity induces expression and secretion of VEGF-A in RPE cells [31]. To determine whether extracellular hyperosmolarity also induces expression of further members of the VEGF family, we performed real-time RT-PCR analysis of RNA extracted from cultured human RPE cells. Cells stimulated with a hyperosmotic medium (+100 mM NaCl) displayed increased expression of VEGF-A, VEGF-D, and PIGF genes, while the expression of VEGF-B and VEGF-C genes was not altered compared to cells cultured in isoosmotic medium (Fig. 1a). High extracellular NaCl induced a dose-dependent increase of the cellular PIGF mRNA level (Fig. 1b). The level of PIGF transcripts was also increased when 100 mM sucrose was added to the medium (Fig. 1c). A decrease of the extracellular osmolarity (60 % osmolarity) resulted in a time-dependent reduction of the cellular level of PIGF gene transcripts (Fig. 1d). The data indicate that the expression of the PIGF gene in RPE cells is dependent on the extracellular osmolarity. Addition of 100 mM NaCl to the culture medium also triggered secretion of PIGF-2 protein from the cells (Fig. 1e). In the presence of high NaCl, the mean PIGF-2 protein content of the cultured media was significantly ( $P < 0.05$ ) increased to  $8.7 \pm 0.2$  pg/ml when compared to unstimulated control ( $7.5 \pm 1.4$  pg/ml; mean  $\pm$  SEM;  $n = 5$ ). As positive control, TGF- $\beta$ 1 was used (Fig. 1e). TGF- $\beta$ 1 is a known inducer of PIGF-2 secretion from RPE cells [14]. In the presence of TGF- $\beta$ 1 (10 ng/ml), the mean PIGF-2 protein content of the cultured media increased to  $9.6 \pm 0.9$  pg/ml ( $P < 0.05$  vs. unstimulated control;  $n = 5$ ).

**Table 1** Primer pairs used in PCR experiments

Gen und Accession nummer	Primer sequenzen (5'→3')	Produkt (bp)
ACTB	s ATGGCCACGGCTGCTTCCAGC	237
NM_001101	as CATGGTGGTGCCGCCAGACAG	
VEGFA <sub>188, 164, 120</sub>	s CCTGGTGGACATCTTCCAGGAGTA	479, 407, 275
NM_003376.5	as CTCACCGCCTCGGCTTGTGACA	
NM_001287044.1		
NM_001025370.2		
VEGFB	s CAGAGGAAACTGGTGTGTCATGGA	233
NM_003377	as ACCGGATCATGAGGATCTGCA	
VEGFC	s CTCTCAAGGCCCAAACCA	152
NM_005429	as AGGTCTTGTTGCTGCCTGA	
VEGFD	s GATCGCTGTTCCCATTTCA	152
NM_004469	as ATCATGTGTGGCCACAGAGA	
PIGF	s GGCGATGAGAATCTGCACTGT	164
NM_001207012.1	as CACCTTCCGGCTTCATCTTC	
FLT1 (VEGFR1)	s CCAAAGCCCCTCTTCCAA	161
NM_002253.2	as CCCCTCTTTCAGCATTTTCCAC	
KDR (VEGFR2)	s CTTTGAAGCATCAGCATAAGAAACT	156
NM_002253	as TGGTCATCAGCCCACTGGAT	
FLT4 (VEGFR3)	s GACGTGGTGAAGATCTGTGACT	227
NM_182925.4	as ACTCCTCATTGATCTGCACCC	
hNRP1	s CCCAGATCACATCATCCAACCA	231
NM_003873	as TGTGCTGTACCCGATCTTGAA	
hNRP2	s TACGATTGGCTGGACATCTGG	170
NM_201266	as AGGTAGTAACGCGCAGAGAAG	

s sense, as anti-sense

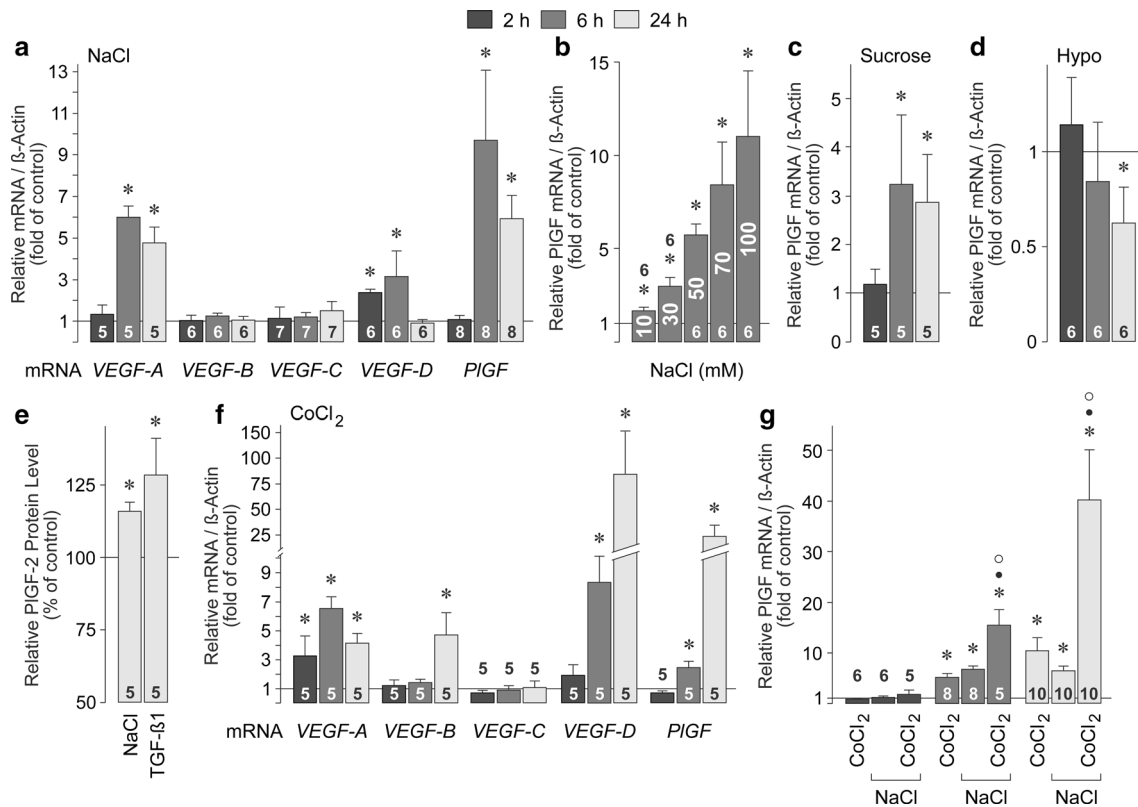
### Hypoxic regulation of VEGF isotype and PIGF genes

In RPE cells, VEGF-A and PIGF are mainly induced by hypoxia [12, 31, 34, 35]. To compare the hypoxic regulation of VEGF isotype and PIGF genes, we performed real-time RT-PCR analysis of RNA from cells cultured in the presence of the hypoxia mimetic CoCl<sub>2</sub> [36]. Chemical hypoxia induced expression of VEGF-A, VEGF-B, VEGF-D, and PIGF genes, while the expression of the VEGF-C gene remained unaltered (Fig. 1f). While increased VEGF-A gene expression was already observed after 2 h of hypoxic stimulation, the hypoxic induction of VEGF-B, VEGF-D, and PIGF gene expression was delayed and greatest after 24 h (Fig. 1f). The data show that hypoxia and high extracellular osmolarity induce expression of VEGF-A, VEGF-D, and PIGF genes in RPE cells.

### Regulation of VEGF and PIGF receptor genes

Human RPE cells were shown to express different VEGF and PIGF receptor genes including VEGF receptor-1 (VEGFR1; the receptor of VEGF-A, VEGF-B, and PIGF),

VEGFR2 (the receptor of VEGF-A, -C, and -D), VEGFR3 (the receptor of VEGF-C and -D), neuropilin-1 (the receptor of specific VEGF-A splice variants, VEGF-B, PIGF-2, and semaphorin-3A), and neuropilin-2 (the receptor of specific VEGF-A splice variants, VEGF-C, PIGF-2, and class 3 semaphorins) [11, 13, 14]. In order to compare the hyperosmotic regulation of VEGF and PIGF receptor genes in RPE cells, we performed real-time RT-PCR analysis of RNA from cells cultured in the presence of high (+ 100 mM) extracellular NaCl. As shown in Fig. 2a, hyperosmotic stimulation induced elevations in the expression of VEGFR2, VEGFR3, and neuropilin-2 genes. The expression of VEGFR1 and neuropilin-1 genes remained unaltered under hyperosmotic conditions (Fig. 2a). Chemical hypoxia induced upregulation of VEGFR2, VEGFR3, and neuropilin-1 genes, while the expression of VEGFR1 and neuropilin-2 genes were not altered (Fig. 2b). The data demonstrate that hypoxia and extracellular hyperosmolarity differentially induce upregulation of PIGF receptor genes in RPE cells, i.e., neuropilin-1 (hypoxia) and neuropilin-2 (hyperosmolarity), respectively.



**Fig. 1** Osmotic and hypoxic regulation of VEGF isotypes and PIGF genes in human RPE cells. mRNA levels in cells cultured 2, 6, and 24 h (as indicated by the panels of the bars), under different conditions were determined with real-time RT-PCR analysis, and are expressed as folds of unstimulated control. The following conditions were tested: hyperosmolarity induced by addition of NaCl (100 mM; **a**) or sucrose (100 mM; **c**) to the culture medium, hypoosmolarity (60 % osmolarity; **d**), and chemical hypoxia induced by addition of CoCl<sub>2</sub> (150  $\mu$ M; **f**). **b** Dose-dependent effect of high extracellular NaCl on the cellular level of PIGF mRNA. Extracellular hyperosmolarity was induced by addition of 10–100 mM NaCl to the culture

medium. **e** Secretion of PIGF-2 protein. The cells were stimulated by addition of NaCl (100 mM) and with TGF- $\beta$ 1 (10 ng/ml), respectively, for 24 h. The protein level in the cultured media was measured by ELISA. Data are expressed as percentage of untreated control (100 %). **g** Effect of coadministration of CoCl<sub>2</sub> (150  $\mu$ M) and NaCl (+100 mM) on the level of PIGF mRNA. The numbers of independent experiments using cell lines derived from different donors are given in or above the bars. Significant difference versus unstimulated control: asterisks  $P < 0.05$ . Significant difference versus NaCl control: filled circle  $P < 0.05$ . Significant difference versus CoCl<sub>2</sub> control: open circle  $P < 0.05$

### Intracellular signaling involved in PIGF expression

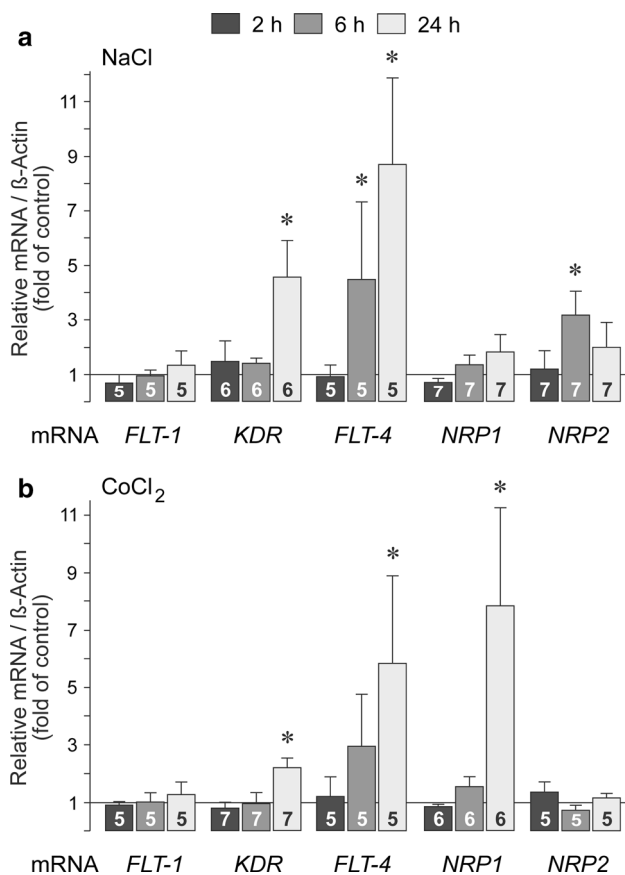
Chemical hypoxia and hyperosmolarity induced additive PIGF gene expression (Fig. 1g), suggesting that different intracellular signaling mechanisms are involved in mediating the gene expression under both conditions. To determine the intracellular signaling mechanisms which mediate the PIGF gene expression, we used inhibitors of key signal transduction pathways. We found that, in dependence on the time period of stimulation, different signal transduction pathways mediate the hyperosmotic PIGF gene expression. After 6 h of stimulation with high extracellular NaCl, the PIGF gene expression was significantly ( $P < 0.05$ ) decreased by inhibitors of the p38 mitogen-activated protein kinase (p38 MAPK; SB203580) and the phosphatidylinositol-3 kinase (PI3K; LY294002), while after 24 h of stimulation, the PIGF mRNA level was reduced by

inhibitors of p38 MAPK (SB203580) and extracellular signal-regulated kinases 1 and 2 (ERK1/2; PD98059) (Fig. 3a). Inhibition of c-Jun NH<sub>2</sub>-terminal kinase (JNK) by SP600125 did not alter the PIGF mRNA level under hyperosmotic conditions (Fig. 3a). The CoCl<sub>2</sub>-induced expression of the PIGF gene was significantly ( $P < 0.05$ ) reduced by the p38 MAPK inhibitor SB203580 after 6 h of stimulation, and by the JNK inhibitor SP600125 after 24 h (Fig. 3b). The hypoxic expression of the PIGF gene was not decreased by inhibitors of ERK1/2 (PD98059) and PI3 K (LY294002) activation (Fig. 3b).

### Receptor signaling involved in PIGF expression

It has been shown that extracellular hyperosmolarity induces secretion of growth factors like VEGF and bFGF from RPE cells [31, 37]. To evaluate an involvement of





**Fig. 2** Hyperosmotic and hypoxic regulation of VEGF and PIGF receptor genes in human RPE cells. mRNA levels in cells cultured 2, 6, and 24 h (as indicated by the panels of the bars) under hyperosmotic (a) and hypoxic conditions (b) were determined with real-time RT-PCR analysis, and are expressed as folds of unstimulated control. Hyperosmolarity and chemical hypoxia were induced by addition of NaCl (100 mM) and CoCl<sub>2</sub> (150 μM) to the culture medium, respectively. The gene expression of following receptor proteins was investigated: VEGFR1 (FLT-1), VEGFR2 (KDR), VEGFR3 (FLT-4), neuropilin-1 (NRP1), and neuropilin-2 (NRP2). The numbers of independent experiments using cell lines derived from different donors are given in the bars. Significant difference versus unstimulated control: \* $P < 0.05$

autocrine/paracrine receptor activation in mediating the PIGF gene expression under hyperosmotic conditions, we used blockers of receptor kinases. The PIGF gene expression induced by stimulation with high NaCl for 6 h was significantly ( $P < 0.05$ ) reduced by the blocker of the platelet-derived growth factor (PDGF) receptor tyrosine kinase, AG1296, and increased by the blocker of the fibroblast growth factor (FGF) receptor kinase, PD173074 (Fig. 4a). The NaCl-induced expression of the PIGF gene was not altered by blockers of VEGF receptor-2 (SU1498), the epidermal growth factor (EGF) receptor tyrosine kinase (AG1478), and TGF-β1 superfamily activin receptor-like kinase receptors (SB431542) (Fig. 4a). None of the

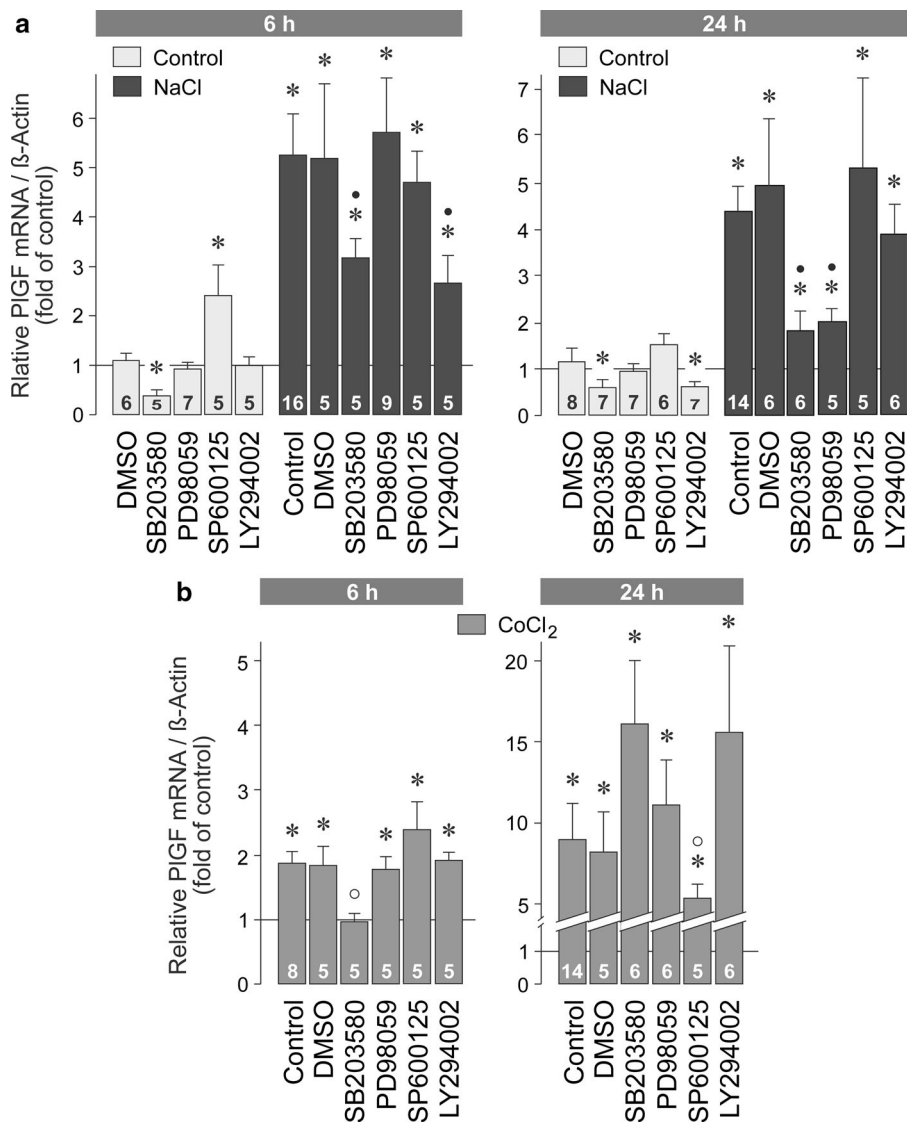
blockers tested decreased the PIGF gene expression in cells stimulated 24 h with high extracellular NaCl (Fig. 4b). The data suggest that, within 6 h of stimulation with high extracellular NaCl, autocrine/paracrine PDGF signaling may be involved in mediating the PIGF gene expression while autocrine/paracrine FGF signaling inhibits the expression. The hypoxic expression of the PIGF gene was significantly ( $P < 0.05$ ) decreased by the inhibitor of TGF-β1 superfamily activin receptor-like kinase receptors, SB431542, after 6 h of stimulation with CoCl<sub>2</sub>, and increased by the VEGF receptor-2 inhibitor SU1498 after 24 h of stimulation (Fig. 4b).

### Role of transcription factors in PIGF expression

It was shown that elevated extracellular osmolarity induces the expression of different transcription factors in RPE cells like hypoxia-inducible transcription factor 1α (HIF-1α), nuclear factor (NF)-κB, and NFAT5 [31]. To determine which transcription factors mediate the hyperosmotic and hypoxic PIGF gene expression, we used pharmacological blockers. The hyperosmotic PIGF gene expression was not altered in the presence of a HIF inhibitor [38], the inhibitor of signal transducer and activator of transcription 3 (STAT3), Stattic [39], and the NF-κB inhibitor CAPE [40] (Fig. 5a). The PIGF gene expression induced by chemical hypoxia was significantly ( $P < 0.05$ ) increased by HIF and NF-κB inhibitors after 6 h of stimulation with CoCl<sub>2</sub>, and decreased by the STAT3 inhibitor Stattic after 24 h (Fig. 5b). Inhibition of NF-κB activity with CAPE triggered a significant ( $P < 0.05$ ) upregulation of the PIGF gene expression under unstimulated control conditions which was observed after 24 h but not after 6 h of stimulation (Fig. 5a).

In different cell systems, the cell survival in hyperosmotic stress is supported by NFAT5 [32]. To determine whether hyperosmotic and hypoxic PIGF gene expression depends on NFAT5 activity, we depleted NFAT5 expression using siRNA. NFAT5 siRNA reduced significantly ( $P < 0.05$ ) the NFAT5 mRNA level by approximately 50 % in cells which were cultured 48 h in control medium (Fig. 6a). Nontargeted siRNA was without effect (Fig. 6a). NFAT5 siRNA also significantly ( $P < 0.05$ ) diminished the hyperosmotic PIGF gene expression by approximately 50 % while nontargeted siRNA had no effect (Fig. 6b). In contrast, NFAT5 siRNA did not alter the upregulation of the PIGF mRNA level induced by chemical hypoxia (Fig. 6c). Similar results were obtained using NFAT5 siRNA from two different commercial suppliers (not shown). The data indicate that the hyperosmotic upregulation of the PIGF gene expression is in part mediated by the activity of NFAT5.

**Fig. 3** Intracellular signaling involved in osmotic (a) and hypoxic (b) expression of the PIGF gene in RPE cells. **a** The level of PIGF mRNA was determined with real-time RT-PCR analysis in cells cultured 6 (left) and 24 h (right) in iso- and hyperosmotic (+100 mM NaCl) media, respectively, and are expressed as folds of unstimulated (isoosmotic) control. **b** The level of PIGF mRNA was determined in cells cultured 6 (left) and 24 h (right) under control conditions and in the presence of  $\text{CoCl}_2$  (150  $\mu\text{M}$ ), respectively. The following pharmacological inhibitors were tested: the inhibitor of p38 MAPK activation, SB203580 (10  $\mu\text{M}$ ), the inhibitor of ERK1/2 activation, PD98059 (20  $\mu\text{M}$ ), the JNK inhibitor SP600125 (10  $\mu\text{M}$ ), and the inhibitor of PI3K-related kinases, LY294002 (5  $\mu\text{M}$ ). Vehicle control was made with dimethylsulfoxide (DMSO; 1:1000). The numbers of independent experiments using cell lines derived from different donors are given in the bars. Significant difference versus unstimulated control: asterisks  $P < 0.05$ . Significant difference versus NaCl control: filled circle  $P < 0.05$ . Significant difference versus  $\text{CoCl}_2$  control: open circle  $P < 0.05$

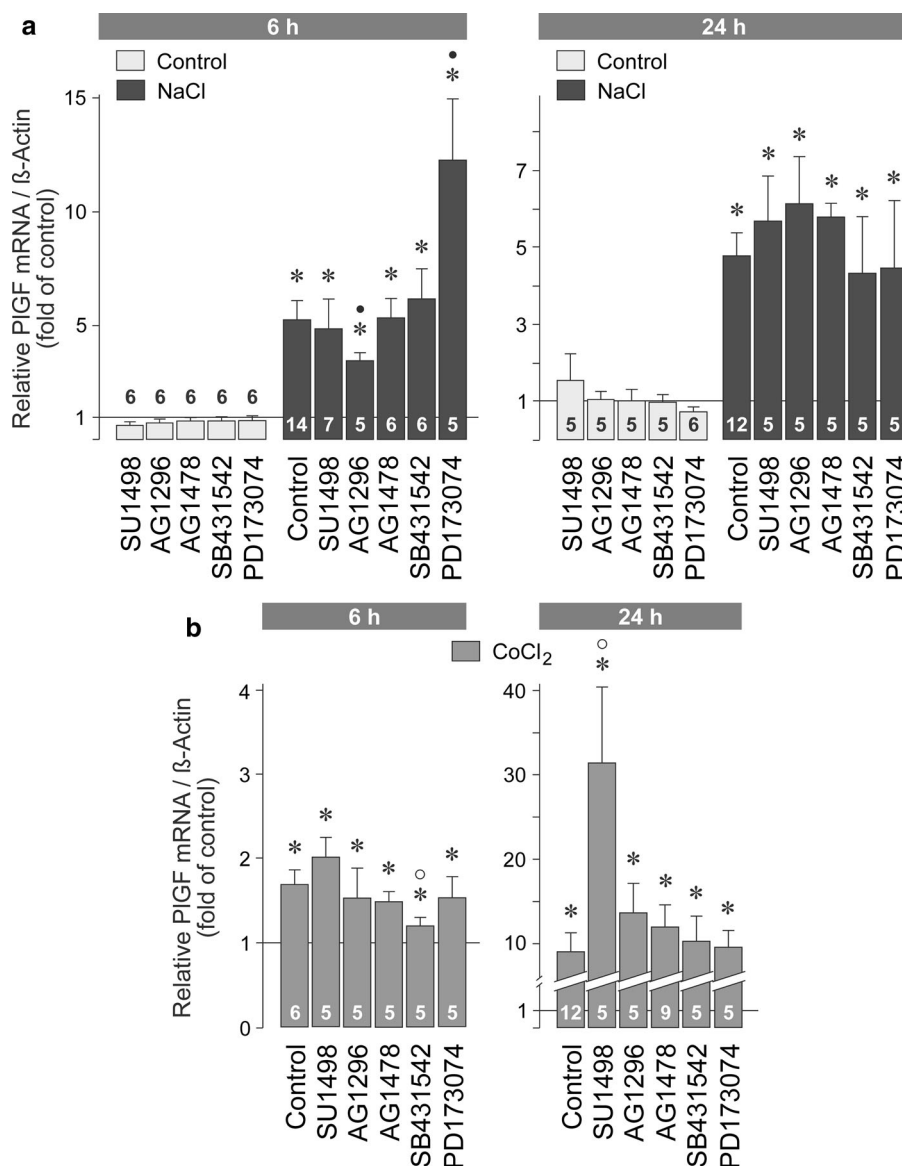


## Discussion

Systemic hypertension is associated with a higher risk of wet AMD [24–26]. On the other hand, the use of antihypertensive medication was shown to be associated with an increased risk of wet AMD [24]. Therefore, hypertension-related conditions, in particular high extracellular osmolarity following the consumption of dietary salt [27], are assumed to aggravate the development of neovascular AMD [31]. It was described that elevation of the extracellular osmolarity induces production of VEGF-A in RPE cells [31], the most relevant angiogenic factor implicated in the development of choroidal neovascularization [5]. Here, we describe that increased extracellular osmolarity also promotes the expression of VEGF-D and PIGF genes in RPE cells (Fig. 1a), and that chemical hypoxia induces the expression of VEGF-A, VEGF-B, VEGF-D, and PIGF

genes (Fig. 1f). Because PIGF gene expression induced by high NaCl and addition of sucrose had similar time dependencies (Fig. 1a and c), PIGF gene expression is rather caused by extracellular hyperosmolarity and not by variation of the transmembrane NaCl gradient. Apparently, the PIGF gene expression in RPE cells is osmolarity-dependent, with up- (Fig. 1a) and downregulation (Fig. 1d) under hyper- and hypoosmotic conditions, respectively. The expression of the PIGF gene triggered by chemical hypoxia and hyperosmolarity was additive (Fig. 1g), suggesting that extracellular hyperosmolarity induces expression of the PIGF gene in RPE cells independently from hypoxia and that different signaling mechanisms are involved in mediating the PIGF gene expression under these conditions.

NaCl-induced extracellular hyperosmolarity induced expression of VEGFR2, VEGFR3, and neuropilin-2 genes



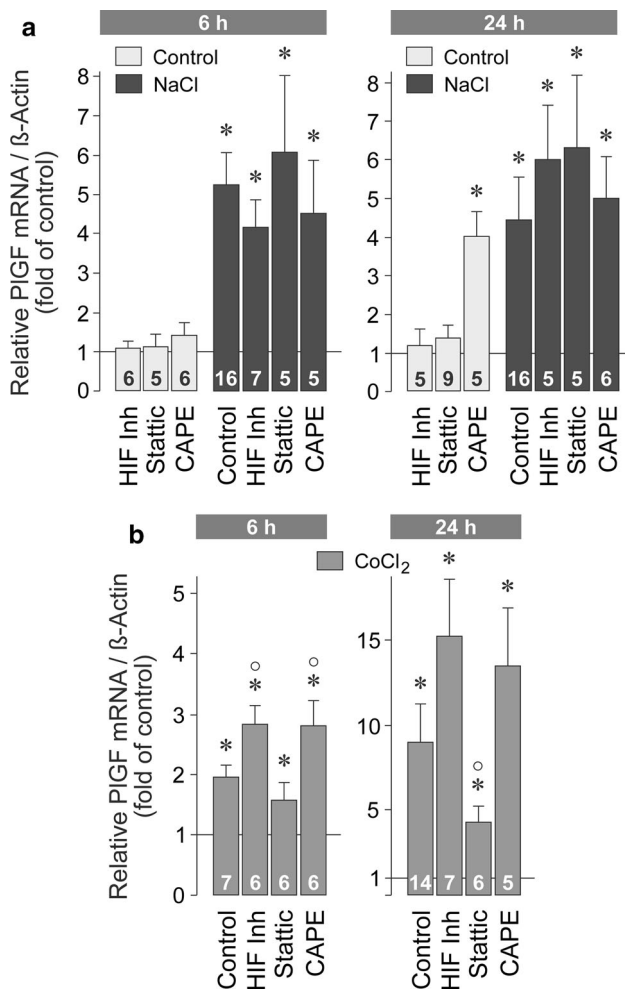
**Fig. 4** Extracellular signaling involved in the osmotic (a) and hypoxic (b) expression of the PIGF gene in RPE cells. **a** The level of PIGF mRNA was determined with real-time RT-PCR analysis in cells cultured 6 (left) and 24 h (right) in iso- (control) and hyperosmotic (+100 mM NaCl) media, respectively, and are expressed as folds of unstimulated (isoosmotic) control. **b** The level of PIGF mRNA was determined in cells cultured 6 (left) and 24 h (right) under control conditions and in the presence of CoCl<sub>2</sub> (150  $\mu$ M), respectively. The following blocking agents were tested: the inhibitor of VEGF receptor-2, SU1498 (10  $\mu$ M), the blocker of the

PDGF receptor tyrosine kinase, AG1296 (10  $\mu$ M), the inhibitor of the EGF receptor tyrosine kinase, AG1478 (600 nM), the inhibitor of TGF- $\beta$ 1 superfamily activin receptor-like kinase receptors, SB431542 (10  $\mu$ M), and the FGF receptor kinase inhibitor, PD173074 (500 nM). The numbers of independent experiments using cell lines derived from different donors are given in or above the bars. Significant difference versus unstimulated control: asterisk  $P < 0.05$ . Significant difference versus NaCl control: filled circle  $P < 0.05$ . Significant difference versus CoCl<sub>2</sub> control: open circle  $P < 0.05$

(Fig. 2a). On the other hand, hypoxia induced expression of VEGFR2, VEGFR3, and neuropilin-1 (Fig. 3B). Neuropilins are receptors of specific VEGF isoforms and PIGF and are thought to act in concert with VEGFRs to modulate angiogenic processes. By complex formation between neuropilins and VEGFRs, neuropilins enhance VEGF activity via VEGFRs. The different expression of both

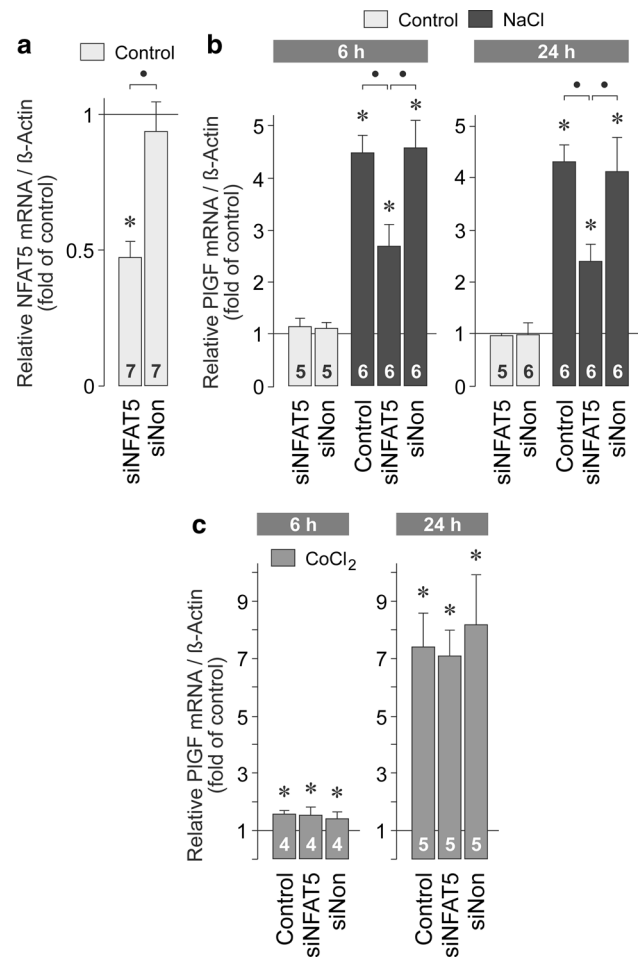
neuropilin genes under hyperosmotic and hypoxic conditions may suggest different functional roles of neuropilin-1 and neuropilin-2 in hypoxia and osmotic stress. Whether the different expression of neuropilins results in distinct regulation of the angiogenic action of VEGF and PIGF under hypoxic and hyperosmotic conditions remains to be investigated in future studies.





**Fig. 5** Involvement of transcription factor activities in osmotic (a) and hypoxic (b) expression of the PIGF gene. **a** The level of PIGF mRNA was determined with real-time RT-PCR analysis in cells cultured 6 (left) and 24 h (right) in iso- (control) and hyperosmotic (+100 mM NaCl) media, respectively, and are expressed as folds of unstimulated (isoosmotic) control. **b** The level of PIGF mRNA was determined in cells cultured 6 (left) and 24 h (right) under control conditions and in the presence of CoCl<sub>2</sub> (150  $\mu$ M), respectively. The following blocking agents were tested: a HIF inhibitor (HIF-Inh; 5  $\mu$ M), the STAT3 inhibitor Stattic (1  $\mu$ M), and the NF- $\kappa$ B inhibitor caffeic acid phenethyl ester (CAPE; 5  $\mu$ M). The numbers of independent experiments using cell lines derived from different donors are given in the bars. Significant difference versus unstimulated control: asterisk  $P < 0.05$ . Significant difference versus CoCl<sub>2</sub> control: open circle  $P < 0.05$

It was shown that hyperosmotic stress activates different protein kinases like p38 MAPK and ERK1/2 in RPE cells, and induces secretion of growth factors such as VEGF and bFGF [31, 37]. We found that, in dependence on the time period of stimulation, activation of different intracellular signal transduction contribute to the hyperosmotic expression of the PIGF gene (Fig. 3a). However, blockers of p38 MAPK, PI3K, and ERK1/2 activation did not completely inhibit the hyperosmotic PIGF gene expression (Fig. 3a),



**Fig. 6** Involvement of NFAT5 activity in osmotic and hypoxic expression of the PIGF gene. The mRNA levels were determined with real-time RT-PCR analysis in cells transfected with NFAT5 siRNA (siNFAT5; 10 nM) and nontargeted siRNA (siNon; 10 nM), respectively, and are expressed as folds of unstimulated (isoosmotic) control. **a** Transfection of RPE cells with NFAT5 siRNA resulted in a reduction of the NFAT5 mRNA level in cells cultured 48 h in isoosmotic control medium. Nontargeted siRNA had no effect. **b** The PIGF mRNA level was determined in cells cultured 6 (left) and 24 h (right) in iso- (control) and hyperosmotic (+100 mM NaCl) media, respectively. **c** The PIGF mRNA level was determined in cells cultured 6 (left) and 24 h (right) in the presence of CoCl<sub>2</sub> (150  $\mu$ M), respectively. siRNA transfection was done 48 h before osmotic (b) or hypoxic (c) stimulation. The numbers of independent experiments using cell lines derived from different donors are given in the bars. Significant difference versus unstimulated control: asterisk  $P < 0.05$ . filled circle  $P < 0.05$

suggesting that further signaling molecules may be involved. We also found that autocrine/paracrine PDGF signaling is required for the full induction of the PIGF gene expression under hyperosmotic conditions whereas FGF receptor signaling inhibits the gene expression under these conditions (Fig. 4a).

There are conflicting data regarding the hypoxic expression of VEGF isoforms and PIGF in the RPE and retina. It was shown, for example, that hypoxia induces

upregulation of VEGF-A and downregulation of VEGF-C and -D in RPE cells [12]. Other studies showed that retinal ischemia induces upregulation VEGF-A and VEGF-C [41], and expression of PIGF protein but not PIGF mRNA [42]. Here, we found that chemical hypoxia induces expression of VEGF-A, VEGF-B, VEGF-D, and PIGF genes in RPE cells (Fig. 1f). The results are in accordance with previous studies that showed that chemical hypoxia induces secretion of VEGF-A, but not VEGF-C, from RPE cells [43], and that hypoxia induces a delayed (15 h) upregulation of PIGF gene expression in RPE cells [35]. While the expression of the VEGF-A gene displayed an immediate upregulation in response to hypoxic stimulation, the expression of the further genes investigated was delayed and displayed a peak after 24 h of stimulation (Fig. 1f). This suggest that different cellular signaling mechanisms are involved in mediating the hypoxic expression of the various genes. We found that the hypoxic expression of the PIGF gene was increased when the VEGF receptor-2 inhibitor SU1498 was present in the culture medium (Fig. 4b), suggesting that autocrine/paracrine VEGF signaling may suppress the hypoxic expression of the PIGF gene. Because we found that a HIF inhibitor increased the hypoxic expression of the PIGF gene (Fig. 5b), it is conceivable that (at least in part) newly synthesized VEGF-A initially suppresses the PIGF gene expression under hypoxic conditions.

It was shown in RPE cells that hyperosmotic stress activates multiple transcription factors like HIF-1 $\alpha$ , NF- $\kappa$ B, and NFAT5 [31]. The hyperosmotic expression of the PIGF gene is in part dependent on the activity of NFAT5 (Fig. 6b) while the hypoxic expression is partly dependent on the activity of STAT3 (Fig. 5b). The latter observation is consistent with a recent study which showed that STAT3 activity induces PIGF gene expression in synovial fibroblasts [44]. Inhibition of the NF- $\kappa$ B activity induced a delayed increase of the PIGF gene expression under control conditions (Fig. 5b). We found a similar CAPE-induced upregulation of distinct other growth factor genes in RPE cells, e.g., of the heparin-binding epidermal growth factor-like growth factor gene (M. Hollborn, unpublished data), but not of VEGF-A [31] and bFGF genes (M. Hollborn, unpublished data). The functional significance of these findings remains to be investigated in future studies. It was found in RPE cells that hyperosmotic stress induces expression of NFAT5, and NFAT5 DNA-binding activity, while hypoxia does not induce NFAT5 gene expression [31]. NFAT5 is the major transcription factor that regulates the cell survival in osmotic stress [32]. NFAT5 activity was shown to contribute to the hyperosmotic expression of the VEGF-A gene in RPE cells [31] and to the neuronal degeneration in the retina of diabetic rats [45]. Here, we found that the hyperosmotic (Fig. 6b), but not the hypoxic

expression (Fig. 6c) of the PIGF gene in RPE cells is in part dependent on the activity of NFAT5. Together with previous data [31], the present results show that NFAT5 activity induces the expression of various angiogenic factors (VEGF-A and PIGF) in RPE cells under osmotic stress conditions; this suggests that NFAT5 may represent a target to treat choroidal neovascularization in wet AMD.

In summary, the present study shows that osmotic stress induces expression of various VEGF isotype and PIGF genes in RPE cells (Fig. 1a), as well as secretion of PIGF-2 from the cells (Fig. 1e). We observed stimulatory effects on the expression of the PIGF gene after addition of 10 mM NaCl to the culture medium (Fig. 1b). It was described that the blood osmolarity in human subjects can increase to values around 360 mosmol/kg under pathological conditions (which can be achieved by adding 40 mM NaCl to the culture medium) [46, 47]. Therefore, the present results may be relevant for the interpretation of in vivo conditions. The data support the assumption that high consumption of dietary salt may result in direct effects on the RPE. Because high extracellular NaCl induces PIGF expression independently from hypoxia (Fig. 1g), high intake of dietary salt may aggravate the angiogenic response of RPE cells in the hypoxic retina. The salt-induced production of PIGF may contribute to the development of choroidal neovascularization in situ, via promotion of the angiogenic effect of VEGF-A. PIGF produced by RPE cells may stimulate various steps in the development of subretinal neovascularization and edema including vascular tube formation [48], breakdown of the outer blood-retinal barrier [35, 49], and infiltration of microglia/macrophages into the neovascular tissue [18]. On the other hand, RPE-derived PIGF may protect retinal neurons and photoreceptors from degeneration [50, 51]. However, it remains to be investigated in future studies whether salt-induced production of PIGF aggravates the development of wet AMD in vivo, and whether a decrease of the salt consumption and an increase of the water intake (which reduces the extracellular osmolarity) may be helpful to protect the aged retina from the progression of AMD.

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