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Psoriasin (S100A7) promotes stress-induced angiogenesis

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What's already known about this topic?

- Psoriasis is characterized by extensive new blood vessel formation.
- Early microvascular modifications in the psoriatic lesion have received limited attention in psoriasis pathogenesis.

What does this study add?

- Psoriasin expression is promoted by ROS and hypoxia in keratinocytes. It regulates the ROS-induced expression of several psoriasis-associated angiogenic factors, and acts on dermal endothelial cells to promote key features of the angiogenic process.

ABSTRACT

Background: Vascular modifications occur early in the development of psoriasis and angiogenesis is one of the key features in the pathogenesis of the disease.

Objectives: We aimed to identify the role of the S100 protein psoriasin in the angiogenesis associated with psoriasis.

Material and methods: The role of psoriasin in mediating angiogenesis was investigated by silencing psoriasin with siRNA and measuring psoriasis-associated angiogenic factors in human epidermal keratinocytes. The secretion of psoriasin and the effect of psoriasin on general regulators of angiogenesis in keratinocytes, and on endothelial cell migration, proliferation, tube formation and production of angiogenic mediators, was evaluated.

Results: The oxidative stress factors ROS and hypoxia induced the expression of psoriasin. Downregulation of psoriasin in keratinocytes using siRNA altered the ROS-induced expression of the psoriasis-associated angiogenic factors vascular endothelial growth factor (VEGF), heparin-binding EGF-like growth factor (HB-EGF), matrix metalloproteinase (MMP)-1 and thrombospondin (THBS)-1. Overexpression of psoriasin altered several regulators of angiogenesis and led to the secretion of psoriasin protein. Treatment with extracellular psoriasin induced proliferation, migration and tube formation in dermal-derived

endothelial cells to a similar extent as VEGF and interleukin (IL)-17, and induced the expression and release of pro-angiogenic mediators. These effects were suggested to be mediated by the PI3K and NFκB pathways.

Conclusion: These findings suggest that psoriasin expression is promoted by oxidative stress in keratinocytes and amplifies the ROS-induced expression of angiogenic factors relevant to psoriasis. Moreover, extracellularly secreted psoriasin may act on dermal endothelial cells to contribute to key features of the angiogenic process.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease characterized by an intense hyperproliferation and a disturbed maturation of keratinocytes, an inflammatory dermal infiltrate and the formation of new blood vessels, in particular alterations of postcapillary venules within the rete ridges [1-4].

Microvascular modifications constitute one of the earliest detectable changes in the development of the psoriatic lesion[5,6]. These modifications include dilatation, hyperpermeability, tortuosity and elongation of dermal blood vessels and proliferation of endothelial cells[7]. The vascular alterations in psoriasis promote skin inflammation through the recruitment of leukocytes to lesional skin[8]. Several pro-angiogenic factors are upregulated in the psoriatic epidermis. Vascular endothelial growth factor (VEGF), a major epidermis-derived growth factor, is strongly upregulated in psoriatic skin[9] and is important in the initiation of the psoriatic phenotype[10]. Interestingly, the increased expression of VEGF is also present in non-lesional skin[11]. Transgenic mice overexpressing VEGF develop an inflammatory skin disorder resembling psoriasis, with an increased density of tortuous capillaries[12]. Many of the treatments used clinically, including tumor necrosis factor (TNF)-α inhibitors, methotrexate and phototherapy, have anti-angiogenic properties[8]. Despite this, the vascular contribution to the pathogenesis of psoriasis has generally received limited attention. In fact, angiogenesis in psoriasis was recently identified as a “research gap” in the field of psoriasis[13].

Psoriasin (S100A7) was originally identified as being highly expressed in psoriatic lesions[14]. It belongs to the S100 family of calcium binding proteins that are involved in several cellular processes including proliferation, differentiation, invasion and metastasis[15,16]. An elevated expression of psoriasin has been detected in some carcinomas of epithelial origin, such as squamous cell carcinoma of the skin and bladder[17,18], as well as in breast cancer, where its expression has been linked to features of poor prognosis[19,20].

In the skin, the expression of psoriasin is especially pronounced in psoriasis, but it is also elevated in other hyperproliferative and inflammatory disorders[21]. Psoriasin has a distinct antimicrobial effect on E. coli[14,22,23] and has been shown to be chemotactic for CD4+ T cells and neutrophils[24]. Psoriasin is induced by several pro-inflammatory cytokines present in psoriatic skin, such as interleukin (IL)-1, TNF-α, IL-17 and IL-22[25].

The high expression of psoriasin in the psoriatic plaque, along with its elevated expression in other conditions characterized by angiogenesis, makes it a relevant protein to study in the context of angiogenesis.

In this study, we aimed to identify the role of psoriasin in the angiogenesis associated with psoriasis.

MATERIAL AND METHODS

Cell culture conditions and treatments

Neonatal human epidermal keratinocytes (HEKn) were cultured in Epilife medium (Cascade Biologics, Paisley, UK) as described[26]. Human embryonic kidney cells (293FT, Invitrogen, Carlsbad, CA) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco). The immortalized, non-transformed keratinocyte cell line N-TERT-2G was grown in keratinocyte serum-free medium medium (KFSM, Gibco) as described[27]. Human dermal-derived microvascular endothelial cells (HMVEC-d) were cultured in endothelial basal medium (EBM) supplemented with endothelial cell growth medium-2 BulletKit (Lonza, Walkersville, MD). Recombinant human psoriasin was purchased from Abnova (Taipei, Taiwan), the VEGF was obtained from Invitrogen and the IL-17 from R&D systems (Minneapolis, MN). The inhibitors LY294002 and CAPE were from Sigma-Aldrich (St Louis, MO).

Induction of cellular stress

To study the effect of stress conditions on psoriasin production, HEKn were seeded at a density of 500,000 cells/T25 culture flask in complete Epilife medium. Following adherence, the medium was replaced with fresh medium containing 150 or 250 µM H_2O_2 (Sigma-Aldrich) for 24-48 hours (n=4), or 500 µM $CoCl_2$ (Sigma-Aldrich) for 24 hours (n=4). To induce hypoxia (n=6), the adhered cells were given fresh medium and were subsequently moved to a hypoxic chamber with 1% O_2 where they remained for 24 hours. Following treatment, cells were harvested and lysed in RLT-buffer (Qiagen, Hilden, Germany) for RNA

extraction. Protein quantification was performed for n=9 in H_2O_2-treated cells and n=7 for hypoxia-treated cells.

Small interfering RNA (siRNA)

Psoriasin siRNA (Pso-siRNA) and a non-targeting siRNA (C-siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HEKn were transfected with siRNA transfection reagent and siRNA in Opti-MEM (Gibco). Cells were stimulated with H_2O_2 24 hours after transfection. The downregulation of psoriasin was confirmed by quantitative real-time (q)PCR. A total of 6 independent experiments were performed.

RNA extraction, cDNA synthesis and qPCR

RNA was prepared using the RNeasy mini kit (Qiagen) and cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). mRNA expression was analyzed with qPCR on a real-time 7500HT sequence detection system with SYBR green detection (Applied Biosystems, Foster City, CA). The primers were previously described[28-32] or were designed using the Primer Express Software v3.0 (Applied Biosystems). All primer sequences are listed in supplemental table S1. GAPDH or RPLP0 were used as housekeeping genes. The relative expression of the target genes to untreated controls was determined using the comparative C_t method.

Cell proliferation and viability

HMVEC-d cells were seeded at 7,500 cells/well in a 96 well plate and were allowed to adhere before treatment. After adherence, the cell culture medium was replaced with basal medium containing either psoriasin (0.15 µg/mL or 1 µg/mL) or VEGF (10 ng/mL). Cells were allowed to proliferate for 24 hours and the proliferation and viability were determined using a CellTiter 96® AQueous One Solution Cell proliferation assay (MTS) (Promega, Madison, WI) (n=5) or PrestoBlue cell viability reagent (Invitrogen) (n=4), according to the manufacturer's instructions. The results were further verified in 12 well plates and by counting after 24, 48 and 72 hours of treatment, which revealed a significant increase in proliferation at 24 h but not at later time points (data not shown). To examine whether VEGF mediated the proliferative response, cells were treated with 0.2 µg/mL VEGF-neutralizing antibody (RnD Systems), followed by treatment with psoriasin or VEGF for 24 hours. The absorbance was recorded at the recommended wavelengths using the VersaMax Microplate

Reader (Molecular Devices, Sunnyvale, CA). The relative absorbance was calculated for treated cells against untreated cells.

Tube formation assay

The formation of HMVEC-d in capillary-like structures was studied on Geltrex-reduced growth factor basement membrane matrix (Invitrogen), according to the manufacturer's instructions. Briefly, a total of 60,000 HMVEC-d cells were seeded onto the matrix and were allowed to adhere. After adherence, cells were treated with psoriasin (0.15 µg/mL or 1 µg/mL; Abnova, Taipei, Taiwan) or VEGF (10 ng/mL). Tube formation was visualized after 18 hours of treatment using an Olympus IX51 inverted microscope and an Olympus DP70 camera (Olympus, Tokyo, Japan). Three independent experiments were performed. Tube formation assays were quantified in ImageJ with the macro Angiongenesis Analyzer (Gilles Carpentier. ImageJ contribution: Angiogenesis Analyzer. ImageJ News, 5 October 2012) for ImageJ, detecting the total length, defined by the sum of length of segments, isolated elements and branches. As these experiments consisted of merely three repeats, statistical comparison of the quantifications was not deemed meaningful.

Scratch migration assay

The ability of psoriasin to mediate the migration of HMVEC-d was evaluated in a scratch migration assay. Cells were seeded densely in complete medium in 96 well plates and were allowed to adhere. When the cells had adhered, the confluent HMVEC-d monolayer was scratched with a pipette tip and washed with PBS to remove debris. The medium was subsequently replaced with basal medium containing psoriasin (0.15 µg/mL or 1 µg/mL) or IL-17 (50 ng/mL), used as a positive control as it is a key cytokine in psoriasis. To inhibit the PI3K and NFκB pathways, the inhibitors LY204002 (5 µM) and CAPE (50 µM) were applied to the relevant wells 45 minutes prior to psoriasin or IL-17 treatment. DMSO treatment was used as diluent control. Cells were allowed to migrate in reduced serum media containing treatment for 18 hours. Migration of HMVEC-d was visualized using an Olympus IX51 inverted microscope and an Olympus DP70 camera (Olympus). A total of three independent experiments were performed. Scratch assays were quantified in ImageJ (Rasband, W.S., ImageJ,U.S.National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016) using the detect edges feature and measuring the mean intensity in the area of the scratch. As these experiments consisted of merely three repeats, statistical comparison of the quantifications was not deemed meaningful.

Directional cloning of the psoriasin sequence

Total RNA was extracted from homogenized human psoriatic skin biopsies and cDNA synthesis was performed. Psoriasin (NM_002963) was amplified by PCR with gene specific primers. The forward primer included a short sequence at the 5'-end (CACC) for directional cloning into the pENTR/D-TOPO vector (Invitrogen). After PCR amplification and gel purification, the psoriasin DNA was cloned into pENTR followed by transformation into One Shot TOP10 chemically competent E. coli (Invitrogen). Insert DNA from positive clones was verified by DNA sequencing with M13 forward (5'GTAAAACGACGGCCAG-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers.

Lentivirus-mediated gene expression

Complementary DNA encoding the psoriasin gene was cloned into the lentiviral expression vectors pLenti6.3/TO/V5-DEST and used to produce infectious lentivirus particles in 293FT cells according to the manufacturer's instructions (Invitrogen). Keratinocytes with constitutive psoriasin expression were generated by infection of N-TERTs with the lentivirus constructs followed by selection with 10 µg/ml blasticidin. N-TERTs were seeded at a density of 5,000 cells/cm^2 and grown to 40% confluence. The medium was then replaced with new medium that was harvested after 24, 48 and 72 hours and cells were lysed with RLT buffer (Qiagen).

Enzyme-linked immunosorbent assay (ELISA)

The secreted levels of psoriasin, VEGF, MMP9 and IL-8 were quantitated using a psoriasin/S100A7 (Circulex, Woburn, MA), VEGF Quantikine (R&D Systems), MMP9 Quantikine (R&D Systems) or IL-8 Quantikine (R&D Systems) ELISA kit, according to the manufacturer's instructions. ELISAs were performed on cell culture supernatant or total protein extract. To obtain total protein cells were lysed in RIPA buffer (Thermo scientific, Rockford, IL) and the protein level was measured with a Bio-Rad protein assay (Hercules, CA). The protein was diluted in assay buffer before the ELISA assay to avoid RIPA interference. The absorbance of the assays was measured on a VersaMax Microplate Reader (Molecular Devices) at the recommended wavelengths.

Gene expression microarray

100 ng RNA extracted from three psoriasin-expressing or control N-TERTs (n=3) after 24 hours of culture was used to generate hybridization probes using the GeneChip® WT PLUS Reagent kit according to the manufacturer's instructions. Hybridization to GeneChip® human transcriptome arrays 2.0 was performed in a GeneChip® hybridization oven, followed by washing and staining in a GeneChip® Fluidics Station 450 and scanning in a GeneChip® Scanner 3000 7G system. Expression Console software (v1.2.1) was used to verify the quality controls. All GeneChip® products were from Affymetrix (Santa Clara, CA). Cel-files generated by the Affymetrix AGCC program were analyzed using Genespring GX software (v13.0; Agilent Technologies, Santa Clara, CA). A Standard Robust Multi-Array Average (RMA) normalization and background correction was performed. The probe sets were filtered on expression with a 20th percentile cut-off. The fold change was computed by the GeneSpring software as a ratio between the psoriasin-overexpressing cells against the control cells. On the probe sets that remained after the filtering, a 1.5 fold change was applied as a cut-off for up- and down-regulated genes. Heatmaps of the differentially regulated genes were constructed based on normalized, filtered and log2-transformed data with Euclidean similarity measure. Probe sets with a fold change of$\geq$1.5 were mapped on the gene ontology (GO) function, selecting for 'regulators of angiogenesis' and 'positive regulators of angiogenesis' as biological functions.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 6 (GraphPad Software, La Jolla, CA) using a paired or unpaired Student's t-test and Wilcoxon's signed rank test. A value of $p \leq 0.05$ was considered statistically significant. The results are presented as the mean ± the standard deviation (SD).

RESULTS

Psoriasin expression is induced by cellular stress in keratinocytes

Pro-angiogenic factors are triggered by stress signals, such as oxidative stress and hypoxia. In psoriasis, the increased production of ROS overrides the effect of endogenous antioxidants[33]. Treatment with H_2O_2 induced the expression of psoriasin in keratinocytes (Figure 1a and 1b),

with the highest induction seen upon treatment with 250 μM H_2O_2 after 48 hours. As expected, H_2O_2 also led to the expression of VEGF (Figure 2b and 1c).

Hypoxia has been shown to induce the production of ROS[34] and a hypoxic state has been suggested in the psoriatic plaque[35]. We therefore cultured HEKn in a hypoxic chamber with 1% O_2 for 24 hours. This culture significantly induced the expression of psoriasin (Figure 1d and 1f), as well as the expression of VEGF, which functioned as a positive control (Figure 1e and 1g). Similarly, the hypoxia-mimicking agent, $CoCl_2$, induced both psoriasin (Figure 1h) and VEGF expression (Figure 1i). Our data demonstrate that the oxidative stress factors H_2O_2, hypoxia and $CoCl_2$, all induce psoriasin expression in keratinocytes.

Psoriasin mediates H_2O_2-induced expression of psoriasis-associated angiogenic factors in keratinocytes

To determine whether psoriasis-associated factors of angiogenesis are affected by the expression of psoriasin, the levels of these factors were measured after treating keratinocytes with H_2O_2. In addition to giving rise to induced psoriasin expression (Figure 2a), this treatment also increased the levels of the pro-angiogenic factors VEGF (Figure 2b), HB-EGF (Figure 2c), MMP-1 (Figure 2d), MMP-9 (Figure 2e) and IL-8 (Figure 2f), whereas the expression of the anti-angiogenic factor THBS-1 (Figure 2g) was reduced. To investigate the specific effect of psoriasin in the H_2O_2-promoted induction of these factors, we downregulated the endogenous expression of psoriasin using siRNA. The efficient downregulation of psoriasin was confirmed by qPCR (Figure 2a). The expression of the pro-angiogenic factors VEGF (Figure 2b), HB-EGF (Figure 2c) and MMP-1 (Figure 2d) was found to be significantly decreased in cells with downregulated psoriasin expression, suggesting that psoriasin participates in their regulation. The expression of MMP-9 (Figure 2e) and IL-8 (Figure 2f) was decreased without reaching statistical significance. Furthermore, the expression of the anti-angiogenic factor THBS-1 was significantly increased in cells with downregulated psoriasin expression. These results suggest that psoriasin amplifies the angiogenic response to H_2O_2 in keratinocytes.

Psoriasin does not influence angiogenic markers by upregulating VEGF receptors

We speculated that psoriasin might influence these angiogenic factors through the induction of the VEGF receptors R1 and -2, since many of these markers are VEGF targets. Treatment with extracellular psoriasin did not induce the mRNA expression of VEGFR1 and -2 in

keratinocytes, nor did the treatment synergize with VEGF to upregulate these receptors (data not shown).

Overexpression of psoriasin leads to secretion of psoriasin into the extracellular space and altered expression of general regulators of angiogenesis

To further determine the effect of psoriasin on angiogenesis and the potential of the protein to influence surrounding cells, we generated keratinocytes with constitutively overexpressed psoriasin. The efficient upregulation of psoriasin expression was confirmed with qPCR (data not shown) and with a psoriasin ELISA which revealed a pronounced psoriasin expression at all the investigated time points (Figure 2h). These results demonstrate not only the successful overexpression of psoriasin in the generated cells but also that psoriasin protein is highly secreted into the extracellular environment. The overexpression of psoriasin was accompanied by an up-regulation of MMP9 (Figure 2i).

To determine the effect of this overexpression on angiogeneic factors, we performed a microarray expression analysis where we searched for general regulators of angiogenesis. A total of 56,372 probe sets were above the 20th percentile of expression on all arrays and were further analyzed. Of these, 283 probe sets had a fold change of ≥1.5 in the comparison between cells with upregulated psoriasin and control cells.

The overexpression of psoriasin induced the upregulation of several factors which by the GO pathways were associated with the regulation of angiogenesis, including the complement component (C)3, IL-1α, IL-1β, MMP9, coagulation factor (F)3, THBS-1 and TNF-α-induced protein (TNFAIP)3. The GO pathways also identified the downregulated gene, cytokeratin (KRT)1, as a regulator of angiogenesis (supplemental figure S1).

Furthermore, several factors that have been suggested to regulate angiogenesis were altered, including MMP10, plasminogen activator urokinase (PLAU) and transforming growth factor (TGF)β2 (supplemental table S2), which suggests that psoriasin affects a broad range of general regulators of angiogenesis.

Psoriasin induces cell proliferation, migration and capillary-like tube formation in dermal endothelial cells

Key steps in the angiogenic process involve endothelial cell proliferation, migration through the extracellular matrix and the differentiation of endothelial cells into a network of tubes.

In order to study the effect of secreted psoriasin on these steps, HMVEC-d were treated with psoriasin and investigated using an MTS and a Presto Blue viability assay to determine

proliferation, a scratch assay to study migration and a tube network formation assay to evaluate angiogenic capacity.

Extracellular psoriasin induced the proliferation of the endothelial cells, as determined by the MTS assay (Figure 3a) and the Presto Blue viability assay (Figure 3b). This effect was found to be independent of VEGF as VEGF-neutralizing antibody did not abolish the proliferation Figure 3c). Psoriasin also caused a pronounced cellular migration of HMVEC-d (Figure 3d), comparable with that induced by the positive control VEGF. Furthermore, psoriasin triggered the formation of tube networks and generated more cell connections on Geltrex matrix than the basal medium control (Figure 3e). In conclusion, our results demonstrate that extracellular psoriasin increases the angiogenic properties of dermal endothelial cells, to an extent comparable to that of extracellular VEGF.

Psoriasin-induced dermal-derived endothelial cell migration is mediated through the PI3K and NFκB pathways

VEGF and IL-17 activate the PI3K and NFκB signaling pathways in endothelial cells and regulate downstream target molecules involved in blood vessel growth and homeostasis[36,37]. To investigate whether these signaling pathways also underlie psoriasin-induced HMVEC-d migration, we examined the effect of inhibitors of PI3K (LY294002) or NFκB (CAPE) following treatment with psoriasin or IL-17. IL-17 was shown to stimulate HMVEC-d migration and was also used as the positive control (Figure 3f). The promoting effect of psoriasin on HMVEC-d migration was effectively blocked by the inhibition of the PI3K (Figure 3g) and NFκB (Figure 3h) pathways. LY294002 and CAPE alone had no effect on HMVEC-d migration; nor did DMSO, used as a diluent control.

Psoriasin induces the expression of angiogenic factors in dermal endothelial cells

To acquire further insights into the mechanisms by which psoriasin increases angiogenesis, we analyzed intracellular angiogenic factors in HMVEC-d cells in response to extracellular psoriasin. We demonstrated a significant increase in the mRNA expression of the pro-angiogenic factors VEGF (Figure 4a) and IL-8 (Figure 4b). Although it did not reach statistical significance, we observed an increase in MMP-1 (Figure 4c) and IL-6 (Figure 4d) expression. The expression of the anti-angiogenic factor THBS-1 was found to be reduced by the treatment with psoriasin (Figure 4e). In addition, treatment with psoriasin upregulated the expression of the psoriasin receptor RAGE, suggesting that psoriasin exposure might further potentiate cell reactivity to the protein (Figure 4f).

Furthermore, IL-8 protein secretion from the HMVEC-d cells was increased after psoriasin treatment as demonstrated by ELISA (Figure 4g). Extracellular psoriasin thus induces the expression and release of pro-angiogenic cytokines in dermal endothelial cells.

DISCUSSION

ROS play an important role in signal transduction and induce the transcription of cytokines, chemokines and growth factors which participate in many biological processes, including angiogenesis[38]. We have previously shown that psoriasin is induced by H_2O_2 in mammary epithelial cells and induces human umbilical vein endothelial cell proliferation[32,39]. We have also demonstrated that the downregulation of psoriasin in a breast cancer cell line with a high endogenous expression of psoriasin led to decreased tumor growth *in vivo*[28]. In line with these findings, we also found the upregulation of MMP-13 and the downregulation of VEGF in cells with reduced psoriasin levels. Moreover, we observed a significant positive correlation between psoriasin expression and blood vessel density, determined by the immunohistochemical analysis of psoriasin and CD31, an endothelial cell-specific marker[28].

In this study, we detected the upregulation of psoriasin by cellular stress in keratinocytes, such as the ROS H_2O_2 and hypoxia. Furthermore, we demonstrate that psoriasin regulates the expression of the psoriasis-associated angiogenic factors VEGF, HB-EGF, IL-8, MMP-1 and MMP-9 in H_2O_2-treated keratinocytes, as indicated by the diminished response to ROS when psoriasin is silenced with siRNA. We have previously shown that psoriasin in itself increases the intracellular levels of ROS in keratinocytes, suggesting a feedback loop where psoriasin is both induced by ROS and amplifies the angiogenic response to ROS[29].

Among the factors that were regulated by psoriasin, VEGF is of particular interest because of its overexpression in psoriatic keratinocytes and its potent mitogenic effect on endothelial cells[6,40]. Extracellular psoriasin also induced the expression of VEGF and IL-8 in dermal endothelial cells, in parallel with an induced cell proliferation. Furthermore, extracellular psoriasin also caused an increase in the expression of the RAGE receptor. As psoriasin is known to be a ligand of RAGE [41], this suggests that the upregulation of this receptor upon psoriasin treatment may potentiate the response to psoriasin in an autocrine fashion.

The regulation of MMPs by psoriasin is also of interest, since they are involved in the proteolysis of the basement membrane that is a critical step during the very early stages of angiogenesis[42]. Similarly, HB-EGF, which is upregulated in the psoriatic lesion[43], is a critical molecular component in wound healing and angiogenesis[44]. The increase in the anti-angiogenic factor THBS-1 expression in H_2O_2-treated keratinocytes with silenced psoriasin expression is in line with previous findings where we have observed a negative correlation between psoriasin and THBS-1 in normal and ductal carcinoma in situ (DCIS) specimens[45]. The microarray analysis of keratinocytes overexpressing psoriasin also suggests that psoriasin affects a broad range of angiogenic factors. We found that psoriasin is highly secreted and leads to the proliferation, migration and differentiation of dermal endothelial cells.

~~The PI3K and NFkB pathways are involved in IL-17-induced angiogenesis and VEGF-induced endothelial cell migration. We found that the inhibition of the PI3K and NFκB pathways in dermal endothelial cells abolished the psoriasin-induced migration, suggesting that the pro-angiogenic effects of VEGF, IL-17 and psoriasin are mediated by the same pathways.~~

In conclusion, our findings demonstrate that psoriasin amplifies the stress-induced angiogenic response in keratinocytes and endothelial cells and is involved in potent pro-angiogenic activity in several crucial steps in the angiogenic process *in vitro*. These findings provide a link between psoriasin and angiogenesis and suggest that the high expression of psoriasin contributes to the increased vascularization in psoriatic epidermis and the progression of the disease. Our findings raise the possibility that psoriasin could be evaluated as a novel anti-angiogenic target in psoriasis.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. Psoriasin is induced by angiogenesis-promoting stress stimuli in keratinocytes. HEKn were either treated with H_2O_2 (150-250 μM) for 48 hours, cultured in hypoxia or treated with the hypoxia-mimicking agent $CoCl_2$ (500 μM), for 24 hours. The expression of psoriasin and VEGF was analyzed using qPCR. H_2O_2 treatment increased the psoriasin mRNA expression compared with untreated cells, (n=4) **(a)** as well as the expression of both psoriasin (n=9) **(b)** and VEGF protein (n=9) **(c)**. Furthermore, culture in hypoxia increased the psoriasin both on the mRNA (n=6) **(d)** and protein level (n=7) **(f)**. Hypoxia also successfully induced the mRNA (n=6) **(e)** and protein (n=7) **(g)** of the positive control VEGF. The treatment of HEKn with $CoCl_2$ induced the expression of psoriasin **(h)** and the expression of VEGF (n=4) **(i)**. Gene expression levels are shown as the relative fold induction compared with untreated controls. GAPDH **(a)** or RPLP0 **(d, e, h, i)** were used as reference genes. Psoriasin protein expression is measured as ng per mg of total protein and VEGF as pg per mg of total protein. Data are presented as the mean +/- SD. *$p<0.05$ and **$p<0.01$.

Figure 2. The downregulation of psoriasin expression in H_2O_2-treated keratinocytes regulates the expression of angiogenic factors. HEKn were transfected with siRNA directed against psoriasin (Pso-siRNA), or negative control siRNA (C-siRNA) and were treated with 250 μM of H_2O_2 for 48 hours. The expression was analyzed by qPCR. The downregulation of psoriasin was confirmed in H_2O_2-treated HEKn **(a)**. The downregulation of psoriasin suppressed the H_2O_2-induced mRNA expression of VEGF **(b)**, HB-EGF **(c)**, MMP-1 **(d)**, MMP-9 **(e)** and IL-8 **(f)** and increased the mRNA expression of THBS-1 **(g)**. The mRNA expression is normalized to GAPDH. The results are shown as the relative expression of treated cells in relation to untreated cells, (n=6). To study the effect of psoriasin on general regulators of angiogenesis, keratinocytes overexpressing psoriasin were generated. The generated keratinocytes (ps-NTERT) secreted markedly higher levels of psoriasin than the control cells at all investigated time points (n=3), **(h)** and a higher level of MMP9

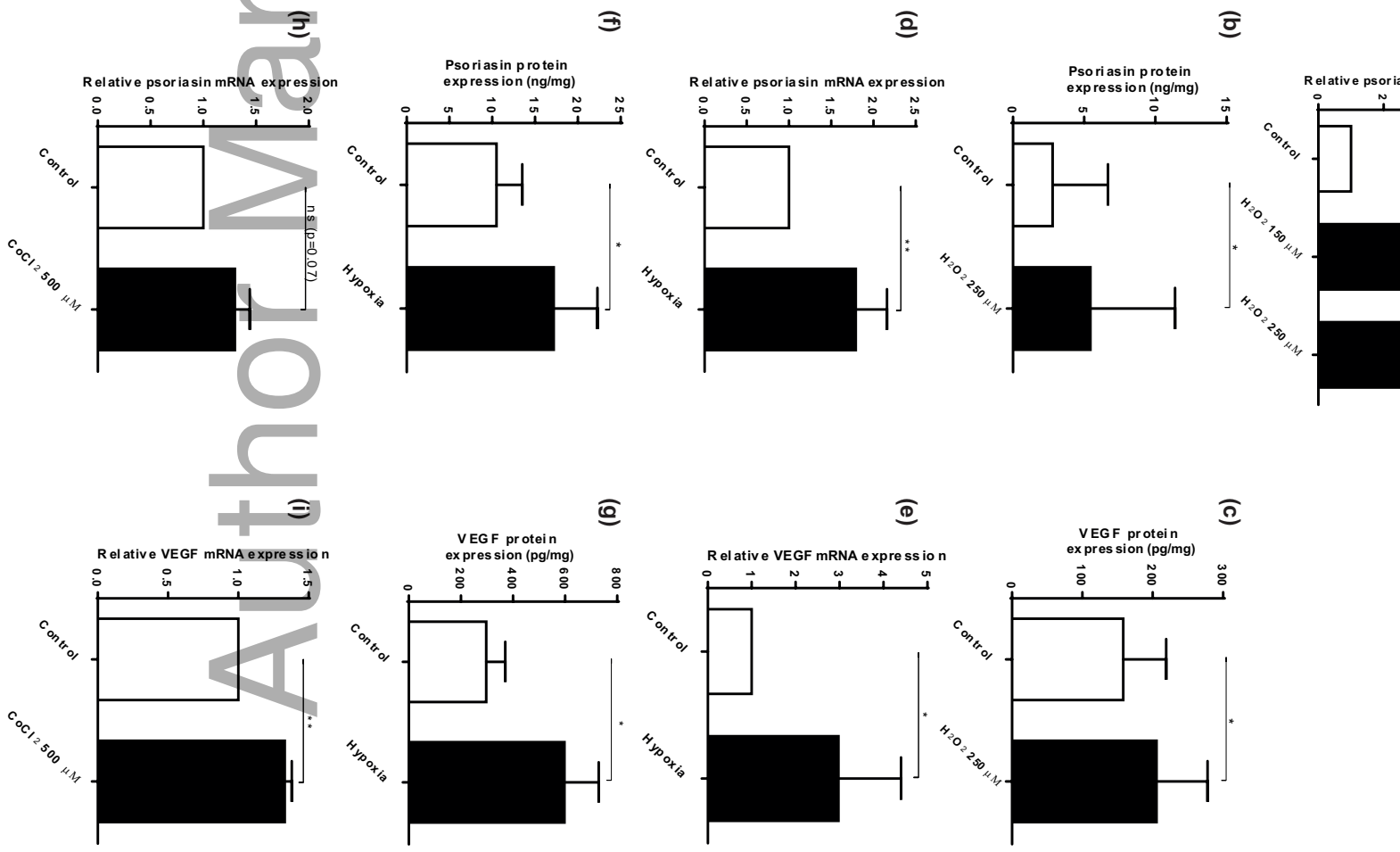
(ng/mL) after 48 h (n=3) **(i)**. Data are expressed as the mean +/- SD, *p<0.05, **p<0.01, ***p<0.001.

Figure 3. Psoriasin induces cell proliferation, migration and the capillary-like tube formation of dermal-derived endothelial cells. HMVEC-d were treated with psoriasin (0.15-1.0 μg/ml) or VEGF (10 ng/ml) for 24 hours. The quantity of viable cells in proliferation was measured by the MTS assay (n=5) **(a)** or the PrestoBlue cell viability reagent (n=4) **(b)**. Treatment with VEGF-neutralizing antibody (0.2 μg/mL) did not abolish the psoriasin-induced proliferation, measured by PrestoBlue (n=7) **(c).** The results are presented as the relative absorbance of the treated cells in relation to the untreated cells. The bar represents the mean +/- SD, *p<0.05 and **p<0.01. In a subsequent experiment, HMVEC-d were grown to confluence and scratched, followed by treatment with psoriasin (0.15-1.0 μg/ml) or VEGF (10 ng/ml) (n=3) **(d)**. HMVEC-d cells were also seeded onto Geltrex and treated with psoriasin or VEGF for 18 hours and capillary-like tube formation was photographed. (n=3) **(e)**. Basal medium served as a negative control, while VEGF and complete medium were used as positive controls.

To investigate the pathways involved in the psoriasin-induced cell migration, HMVEC-d were grown to confluence, scratched and pretreated with LY204002 (5 μM) or CAPE (50 μM) for 45 minutes followed by stimulation with psoriasin or the positive control IL-17 (50 ng/ml) for 18 hours. Psoriasin and IL-17 induced HMVEC-d migration **(f)**. The PI3K-inhibitor, LY204002, **(g)** and the NFκB-inhibitor, CAPE, **(h)** prevented psoriasin-induced migration. Neither LY294002 and CAPE alone, nor DMSO used as a diluent control, had any effect on HMVEC-d migration. The photographs are representative examples of three individual experiments. Bar graphs display the mean +/- SD and display the migration or total length tube formation relative the control.

Figure 4. Psoriasin induces the expression of angiogenic factors in dermal endothelial cells. HMVEC-d were treated with psoriasin (0.15-1.0 μg/ml) for 24 hours. The expression was analyzed by real-time qPCR. Psoriasin treatment induced the mRNA expression of VEGF (n=8) **(a)**, IL-8 (n=8) **(b)**, MMP-1 (n=3) **(c)** and IL-6 (n=8) **(d),** while reducing the mRNA expression of THBS-1 (n=6) **(e)**. Furthermore, the expression of the psoriasin receptor RAGE was upregulated upon treatment (n=6) **(f)**. Treatment with psoriasin also caused the production of IL-8, measured in ELISA in cell supernatant (n=7) **(g)**. VEGF (10 ng/ml) was used as the positive control. The results are shown as the relative expression of

treated cells in relation to untreated cells. The mRNA expression is normalized to GAPDH as the internal control. Data are expressed as the mean +/- SD, *$p<0.05$ and **$p<0.01$.



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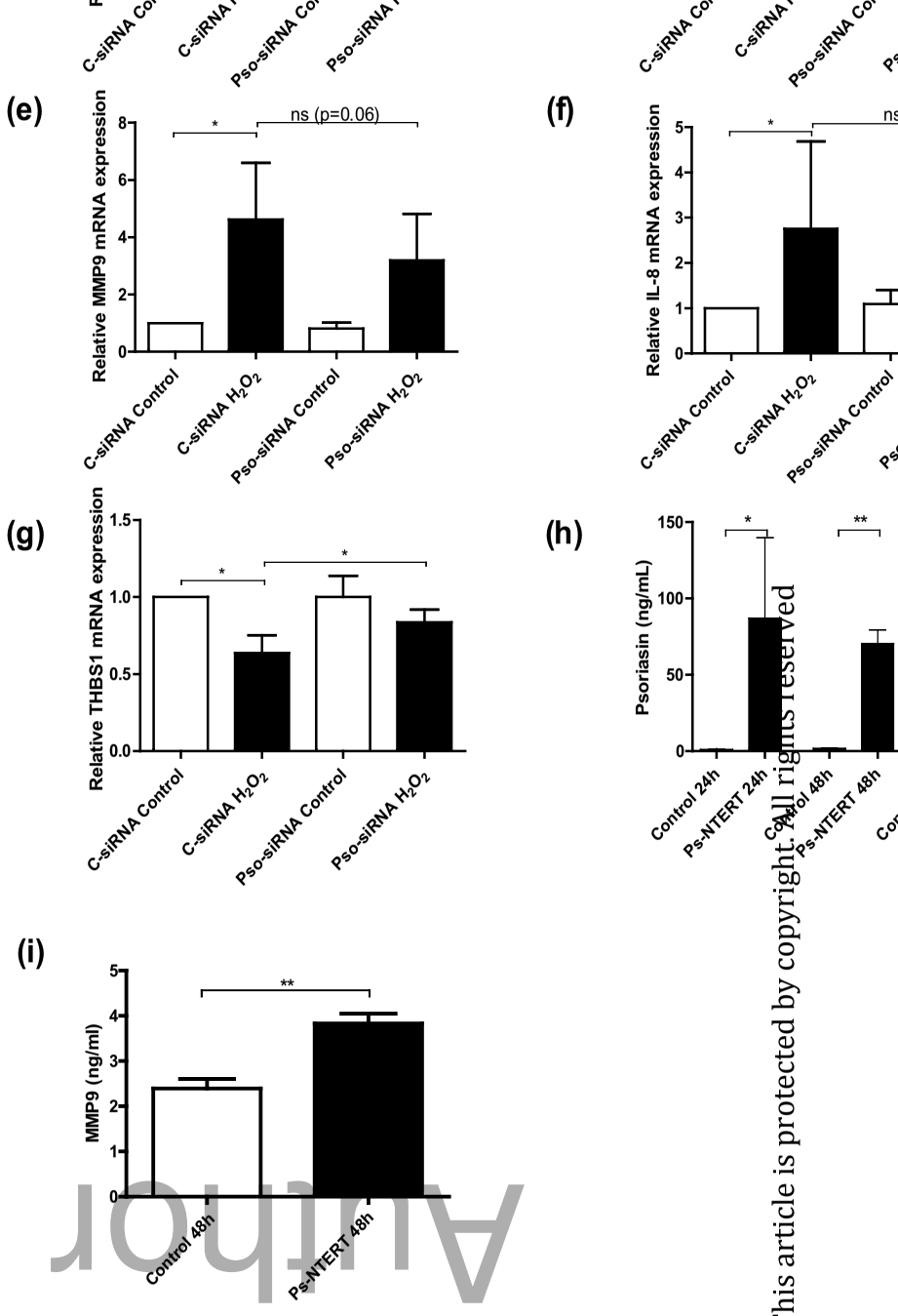

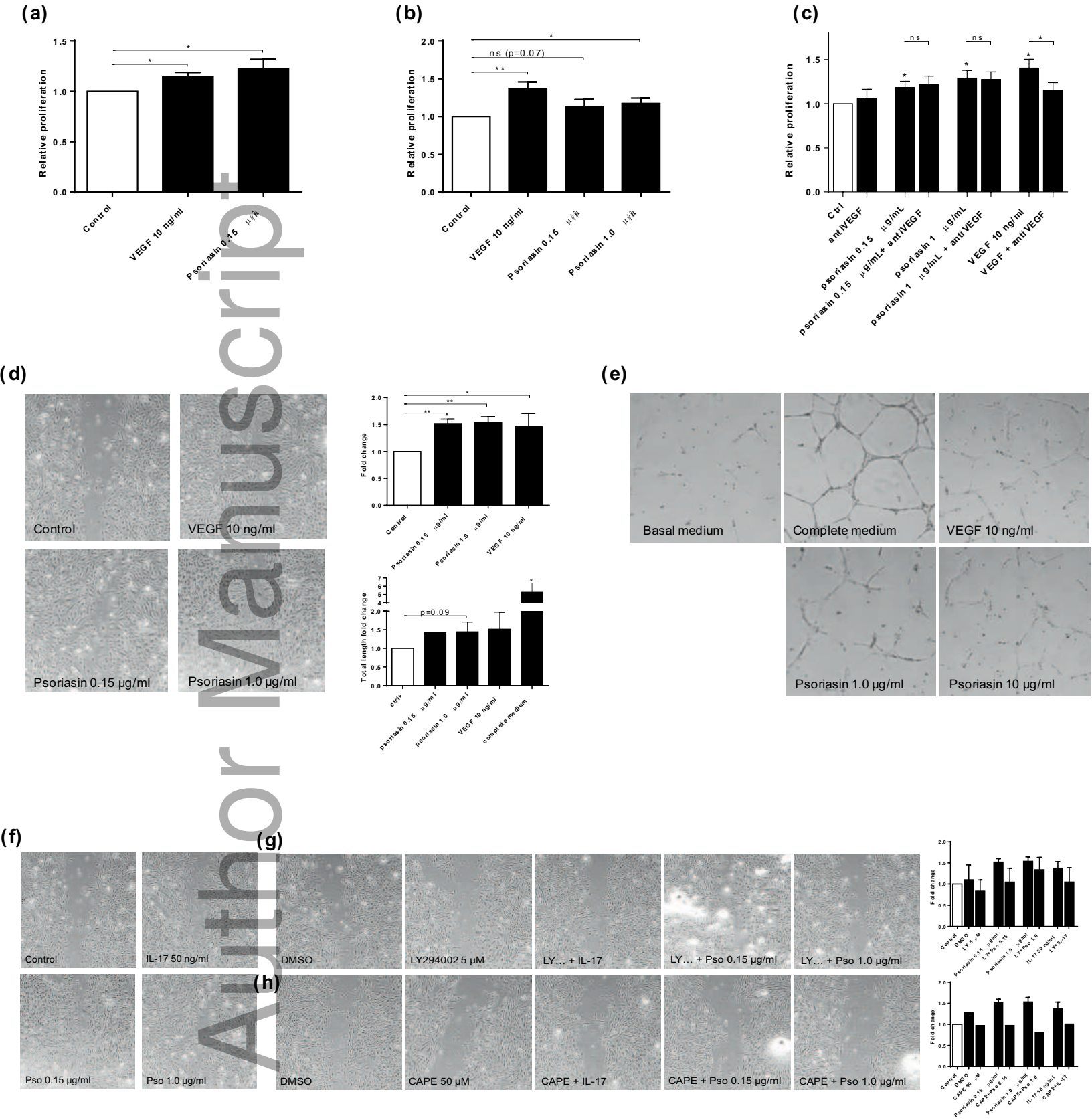

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