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Cold atmospheric plasma (CAP) activates angiogenesis-related molecules in skin keratinocytes, fibroblasts and endothelial cells and improves wound angiogenesis in an autocrine and paracrine mode

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ABSTRACT

Background: Cold atmospheric plasma (CAP) emerged as a novel therapeutic field with applications developed for bacterial sterilization, wound healing and cancer treatment. For clinical implementation it is important to know how CAP works and which molecular changes occur after the CAP treatment. Vascularization is an important step during wound healing, however, the effects of CAP on wound angiogenesis are not well examined so far. Furthermore, it has not been investigated, whether CAP primarily affects endothelial cells directly or via paracrine mechanisms to modulate the vasculature.
Objective: This study concentrates on the influence of CAP on angiogenesis-related molecules in human epidermal keratinocytes, dermal fibroblasts and endothelial cells.
Methods: CAP was generated by the MicroPlaSter ß® plasma torch system and CAP effects on angiogenesis were determined *in vitro* and *in vivo*.
Results: We observed that CAP significantly induces the expression of Artemin, EGF, EG-VEGF (PK1), Endothelin-1 (ET-1), FGF-2 (FGF basic), IL-8 (CXCL8) and uPA in keratinocytes and Angiogenin (ANG), Endostatin (Col18A1), MCP-1 (CCL2), MMP-9, TIMP-1, uPA and VEGF in fibroblasts. In addition, CAP activates the expression of Angiopoietin-2 (Ang-2), Angiostatin (PLG), Amphiregulin (AR), Endostatin, FGF-2 and angiogenic-involved receptor expression of FGF R1 and VEGF R1 in HUVEC endothelial cells. It was also demonstrated that supernatants collected from CAP activated fibroblasts and keratinocytes elevate tube formation by endothelial cells and FGF-2 appears to be an important pro-angiogenic factor that controls vascularization via paracrine mechanisms. Mouse experiments supplement that CAP promotes angiogenesis during wound healing *in vivo*.
Conclusions: Taken together, these results suggest that CAP modulates angiogenesis-involved factors via autocrine and paracrine mechanisms and may be used to affect angiogenesis during wound healing.

1. Introduction

Cold atmospheric plasma (CAP) devices are currently being developed for a variety of medical applications and have already shown efficacy for the treatment of chronic and acute wounds in patients accelerating wound healing by activation of wound healing relevant growth factors and cytokines and by killing bacteria. CAP has also revealed antitumoral effects *in vitro* by inducing malignant cell apoptosis [1–3].

Angiogenesis, the growth of new blood vessels from existing vessels, is a physiological process important not only in embryogenesis but also during growth and wound healing [4,5]. Insufficient vascularization results in impaired wound healing in patients with diabetes [6] and systemic sclerosis [7], and patients treated with immunosuppressive drugs [8], chemotherapy [9] or radiotherapy [10]. Formation of new blood vessels is stimulated by a lack of oxygen and different endogenous pro-angiogenic factors. Among them are not only growth factors (e.g. VEGF, EGF, FGF, TGF) and cytokines (e.g. IL-1, 2, 6, 8; TNF) but also reactive oxygen species (ROS) and reactive nitrogen species (RNS). Since CAP generates different ROS and RNS, it was speculated that CAP could be able to stimulate angiogenesis.

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Indeed, few studies already described a positive effect of CAP on wound angiogenesis either by release of fibroblast growth factor-2 (FGF-2) [11] or by production of NO [12]. Enhanced tube formation by using primary porcine aortic endothelial cells was found by Arjunan et al. [13], who reported that particularly hydroxyl radicals and hydrogen peroxide seem to be responsible for the observed effects [14]. Even in more complex models to determine the influence of CAP on the formation of new micro-vessels, like the in-ovo chick embryo chorioallantoic membrane assay (CAM assay), stimulation of angiogenesis was found [15].

However, the exact mechanisms how CAP activates the vasculature is so far unclear.

We used the MicroPlaSter ß® and analyzed CAP effects on angiogenesis-related molecules in different skin cells and analyzed effects on angiogenesis *in vitro* and *in vivo*.

Understanding the mechanisms of CAP-cell interactions is absolutely necessary and crucial to assure safety during the CAP treatment. Furthermore, it has not been investigated so far, whether CAP primarily affects endothelial cells directly or via paracrine mechanisms to modulate the vasculature.

2. Materials and methods

2.1. Plasma device and treatment of cells

The CAP device used in this study was the MicroPlaSter ß® plasma torch system (Microwave 2.45 GHz, 80 W, argon flow 4.0 l/min, treatment diameter ~5 cm) developed by the Max Planck Institute for Extraterrestrial Physics in Garching/Germany and built by Adtec Plasma Technology Co. Ltd., Hiroshima/London. This device was successfully used in clinical studies for the treatment of chronic and acute wounds [16–18], for skin pruritus [19] and for different *in vitro* and *in vivo* animal models [20,21]. The device is available since November 2014 as CE-certificated medical product under the designation Adtec SteriPlas® (Adtec Healthcare, Houndslow, Middlesex). A description of the plasma torch, plasma device and optical emission spectrum of the plasma discharge can be found in [21]. Treatment of keratinocytes, fibroblasts and endothelial cells with CAP was performed in analogy to our previous study [21].

2.2. Cell culture conditions

Primary human skin keratinocytes were purchased from Cascade Biologics (Eugene, OR, USA; Lot: 5C0733) and ATCC (Manassas, VA, USA; Lot: 58732331 and Lot: 60245430), primary human dermal fibroblasts (2F0621, 9F0438, 9F0889) were purchased from Lonza (Verviers, Belgium) and were cultured according to [20,21]. HUVECs (Human Umbilical Vein Endothelial Cells) were ordered via Gibco (Life Technologies) and were cultured in Medium 200 with LVES (Large Vessel Endothelial Supplement) (both from Life Technologies). All cells were split 1:5 at confluence. Cells in culture were tested all three months for mycobacterial contamination using the PCR Mycoplasma Test Kit (PanReac, AppliChem, Darmstadt, Germany) according to the manufacturer's instruction.

2.3. Human angiogenesis antibody array

A Proteome Profiler™ Human Angiogenesis Antibody Array (array kit ARY007, R&D systems, Minneapolis, USA) was used according to the instruction. Within this array the relative levels of 55 angiogenesis-related proteins in cell culture supernatants from fibroblasts and keratinocytes were analyzed 24 h after 2 min of CAP exposure or from untreated control cells. From HUVECs supernatants were collected 24 h after 30 s of CAP exposure due to a higher CAP sensibility towards endothelial cells. Analytics include soluble growth and differentiation factors, extracellular matrix components, proteases, membrane-bound receptors and intracellular signaling molecules. The arrays were hybridized with an equal amount of total protein (200 µg, quantified using Pierce BCA-kit, Thermo Scientific, Rockford, USA) from CAP or untreated control cell culture supernatants. Detection and densitometry of the array spots was performed according to [21]. Arrays were performed in duplicates with three different fibroblast and keratinocyte cultures and with HUVECs.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants from keratinocytes and fibroblasts were collected 24 h and 48 h after a 2 min CAP treatment or from untreated control cells and were analyzed by ELISAs, respectively, according to [21]. ELISAs for the detection of FGF-2, MCP-1, EG-VEGF and Artemin were all received from R&D Systems, Wiesbaden-Nordenstadt, Germany. The ELISA for the detection of FGFR1 in HUVEC cell lysates was obtained from Cell Signaling and was performed according to the manufacturer's instructions. For the detection of VEGF R1 in HUVEC cell lysates the human VEGFR1/Flt-1 DuoSet ELISA from R&D Systems (DY321B) was used and performed as described. Each sample was assayed in duplicates, and all experiments were performed two times.

2.5. Analysis of mRNA expression by quantitative RT-PCR

Isolation of total cellular RNA 6 h and 24 h after the CAP treatment and reverse transcriptase reaction was performed according to [21] and [22]. Quantitative RT-PCR was performed with specific sets of primers and conditions listed in table S.1 applying LightCycler technology (Roche Diagnostics, Mannheim, Germany) as described [22]. Each RT-PCR was performed in duplicates with cDNA of at least three different keratinocyte and three different fibroblast cell cultures and HUVECs.

2.6. Tube formation assay

For analyzing direct CAP effects 50×10^3 HUVECs were seeded into a 6-well plate and were treated with CAP for 30 s, 1 min and 2 min or remain untreated. Matrigel (Corning® Matrigel® Basement Membrane Matrix) was added into the lower compartment of a 15-well Angiogenesis µ-Slide from Ibidi (Martinsried, Germany) and allowed to gelatinize for 20 min at 37 °C. 15×10^3 CAP-treated or untreated HUVECs in 50 µl medium (Medium 200 with LVES) were transferred immediately into the upper compartment of the µ-Slide.

For analyzing indirect CAP effects HUVECs were seeded into the upper compartment either in 50 µl conditioned medium (Medium 200 with LVES) from keratinocytes or fibroblasts collected 24 h after cells were treated with CAP for 1 min and 2 min or from untreated cells.

To determine FGF-2 specific effects, conditioned medium was supplemented with FGF-2 neutralizing antibody (Anti-FGF-2/basic FGF (neutralizing), clone bFM-1; Merck Millipore, Darmstadt, Germany) in concentrations as indicated.

Tube formation was assessed by phase contrast microscopy after 24 h and recorded with a digital camera (Leica MC170HD). Each experiment contains triplicates and pictures were taken from each of the triplicates using 5 x magnification. Numbers of tubes from all pictures were counted and results were summarized. Experiments were repeated three times.

2.7. Mouse experiments

For the experiments 129 Sv/Ev female mice between 8 and 12 weeks of age were used. The mice were anesthetized, animals' backs were shaved and 6 mm full-thickness wounds were generated using a 6 mm dermal biopsy punch (Stiefel, GSK company, Germany) as described [21]. Wounds of four mice became a daily CAP treatment using the MicroPlaSter ß® (80 W, argon flow 4 l/min) for 2 min, 10 days long. Wounds of control mice were treated with the placebo modus of the MicroPlaSter ß® for 2 min instead of CAP, to resemble the same stress-situation for all animals. All mice were sacrificed on day 15 after wounding and the treated skin area was excised and analyzed as described [21].

Preparation of dermal RNA was performed according to [21] and mRNA expression was analyzed by quantitative RT-PCR as described above. All animal experiments were conducted with appropriate permission from the animal rights commission of the state of Bavaria and maintained in agreement with the European Union guidelines. All experiments were approved by the Committee on the Ethics of Animal Experiments of the University of Regensburg, Germany (Permit Number: 54-2532.1-10/11).

2.8. Immunohistochemistry

Immunohistochemically staining was performed on 4 µm formalin-fixed, paraffin-embedded skin sections. Endogenous

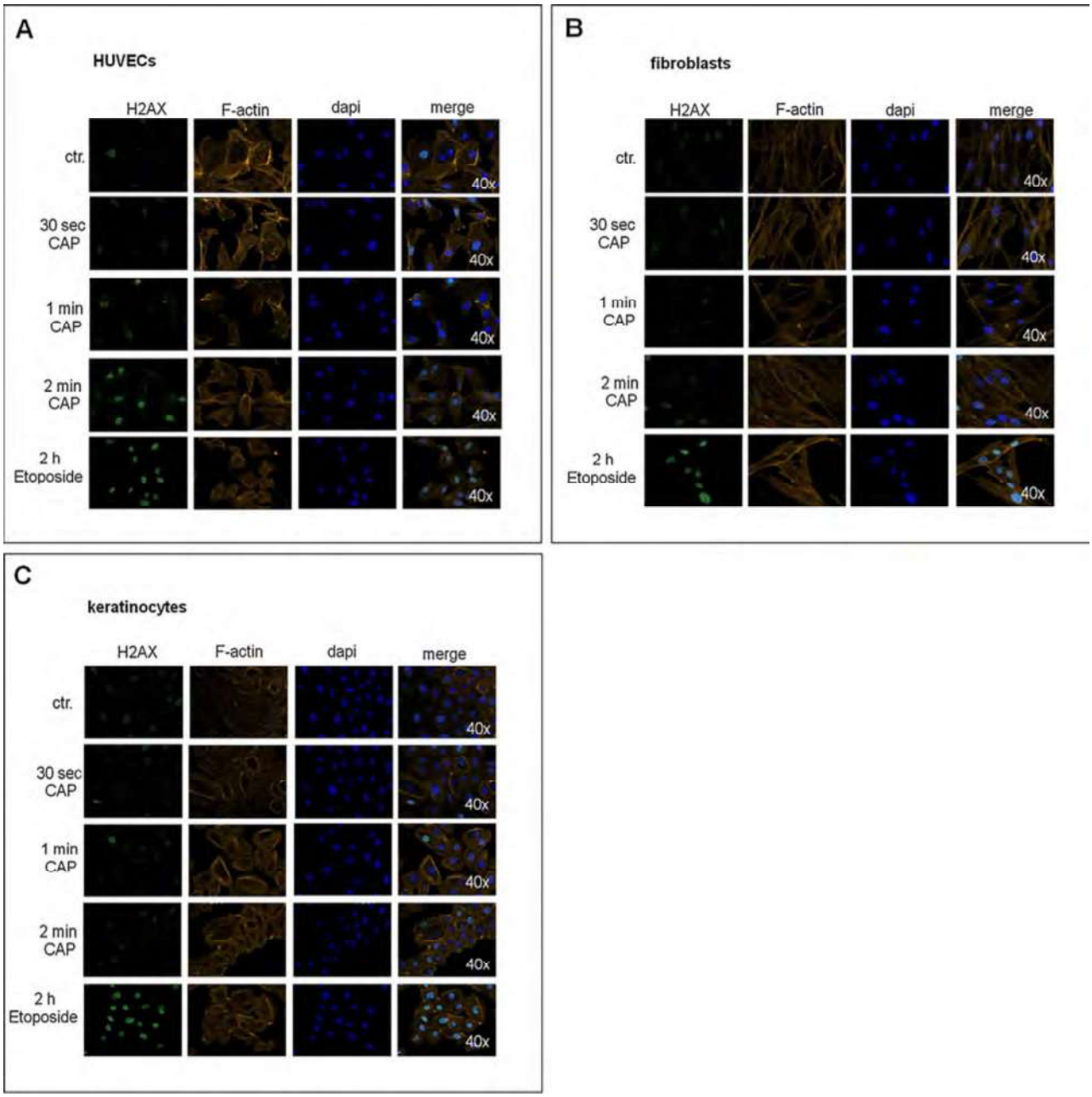

Fig. 1. Analyzation of DNA fragmentation after CAP treatment.
(A) Phosphorylated H2AX (green), F-actin (orange), and dapi (blue) immunofluorescence staining were performed 2 h after treatment with 50 µM etoposide or CAP exposure for 30 s, 1 min and 2 min. H2AX images indicate high amounts of double-strand breaks (DSBs) in HUVECs after etoposide treatment. Similar DSBs were observed after treatment with CAP for 1 min and 2 min in HUVECs. Only little phosphorylation of H2AX was detected after 30 s, 1 min and 2 min CAP exposure in fibroblasts (B) and keratinocytes (C), revealing low frequency of DSBs. Images were collected by immunofluorescence microscopy using 40 x magnification.

peroxidase activity was blocked with 3% hydrogen peroxide (Dako Cytomation GmbH, Hamburg, Germany). Antigen retrieval was carried out in Tris-EDTA buffer (1 mM, pH 8.5) for 5 min at 120 °C in a pressure cooker. Anti-CD31 antibody (Zytomed, Berlin, Germany; ready to use) was incubated for 1 h followed by 30 min incubation with the secondary antibody (Zytomed), 30 min incubation with HRP-conjugate (Zytomed) and reaction was visualized with 3-Amino-9-*E*thylcarbazole (AEC) (Dako) followed by counterstaining with hematoxylin (Merk, Darmstadt, Germany).

Evaluation of the staining was performed semi-quantitatively by means of light microscopy (Carl Zeiss Vision, Hallbergmoos, Germany). Scale bar = 50 μm.

2.9. Immunofluorescence

5×10^3 keratinocytes, fibroblasts and HUVECs/well were seeded into 8-well chamber slides and treated with CAP for 30 s, 1 min and 2 min 24 h after the treatment, cells were washed, fixed, permeabilized and blocked as described previously [23]. As positive control cells were incubated with etoposide (50 μM, Sigma, Taufkirchen, Germany) and incubated for 2 h prior to fixation. Subsequently, cells were incubated with anti-H2AX (phospho-) antibody (1:200; Life Technologies GmbH, Darmstadt, Germany) overnight at 4 °C. After washing, cells on coverslips were incubated with the secondary anti-body (1:40, fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immuno-globulin; from DAKO) for 1 h, followed by rinsing with PBS and co-staining with 80 nmol/L rhodamine phalloidin (F-actin) (Cytoskeleton, Denver, CO, USA) in PBS for 30 min. After washing with PBS, cells were mounted with Vectashield Hard Set Mounting Medium with DAPI H-1500 (Vector Laboratories, Burlingame, CA, USA). Images were collected by immunofluorescence microscopy using an Axio Imager Zeiss Z1 fluorescence microscope (Axiovision Rel. 4.6.3, Carl Zeiss AG, Oberkochen, Germany) with 40 x magnification.

2.10. Statistical analysis

Results are expressed as the mean ± SD. Comparisons between groups were made using Student's unpaired *t*-test. A p value < 0.05 was considered statistically significant ($^*p < 0.05$). All calculations were performed using the GraphPad Prism software package (GraphPad Software Inc., San Diego, CA, USA).

3. Results

In the present study, we used different approaches to determine key players and molecular mechanisms affecting angiogenesis influenced by CAP treatment. Hereby we focused on direct CAP effects on endothelial cells and effects affecting angiogenesis via paracrine mechanisms triggered by CAP treated keratinocytes or fibroblasts.

We chose the MicroPlaSter ß® for our experiments, since several published clinical studies on humans with e.g. acute or chronic wounds already showed a beneficial effect of this specific plasma source [2,17–19,24] and also in *in vitro* and *in vivo* animal models positive effects on wound healing using this plasma source were observed [20,21].

3.1. Endothelial cells are more sensitive towards CAP treatment than keratinocytes and fibroblasts

In the present study we started to analyze direct CAP effects on endothelial cells (HUVECs) to get an impression on the sensitivity of this cell type towards CAP treatment using the MicroPlaSter ß®.

Immunofluorescence-stained gamma H2AX foci were analyzed in HUVECs, fibroblasts and keratinocytes after CAP treatment for 30 s, 1 min and 2 min and further incubation for 24 h compared with the 50 μM etoposide treatment for 2 h. Etoposide is a cancer

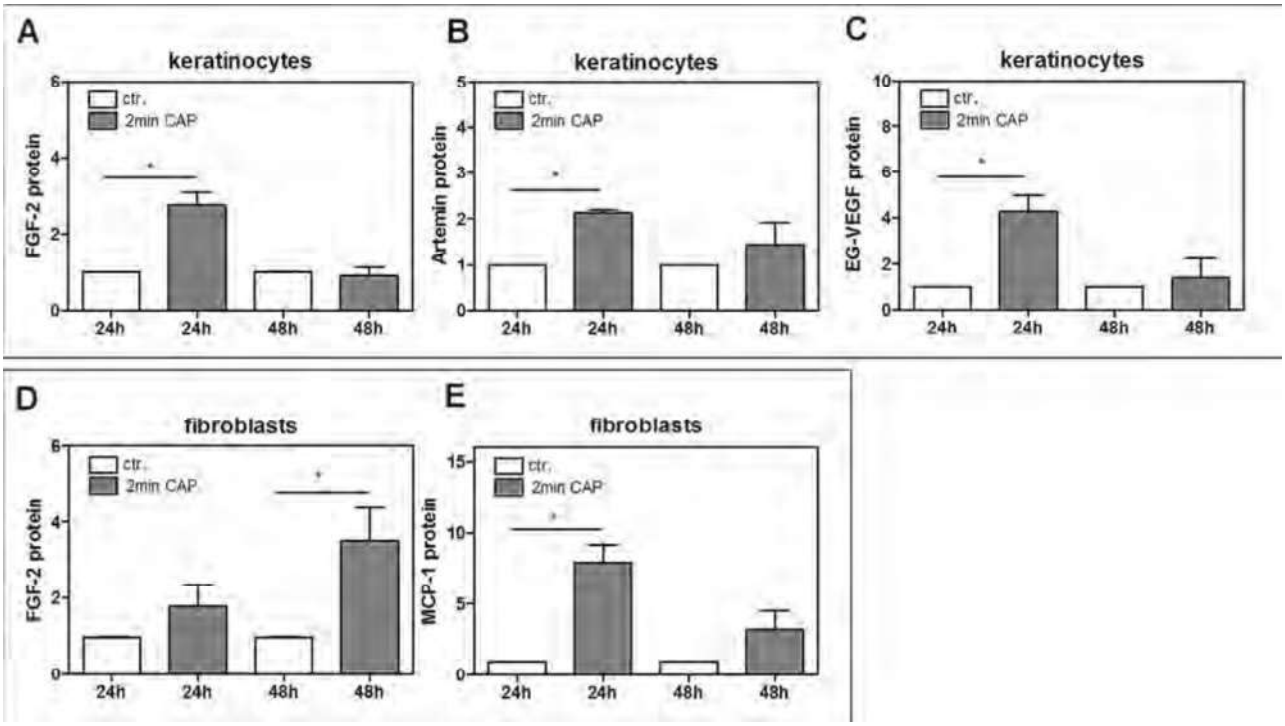

Fig. 2. Secretion of pro-angiogenic factors in keratinocytes and fibroblasts after CAP treatment.
(A–C) Protein secretion of FGF-2, Artemin and EG-VEGF in keratinocytes and FGF-2 and MCP-1 in fibroblasts (D–E) was analyzed 24 h and 48 h after CAP treatment for 2 min by ELISA technique and was compared to the corresponding untreated control (ctr.). $^*p < 0.05$.

chemotherapeutic drug that induces DNA double-strand breaks (DSBs). The level of gamma-H2AX staining after 2 min of CAP treatment in HUVECs was similar to the etoposide treatment, suggesting a strong plasma-induced DSB formation in endothelial cells using high CAP doses (Fig. 1A). Fibroblasts and keratinocytes are less sensitive to high CAP doses. Only a few cells show DSBs after CAP treatments for 2 min (Fig. 1B and C). In summary, these results suggest that endothelial cells are more sensitive, wherefore we decided to adjust the CAP treatment time to 30 s for all direct endothelial cell treatments.

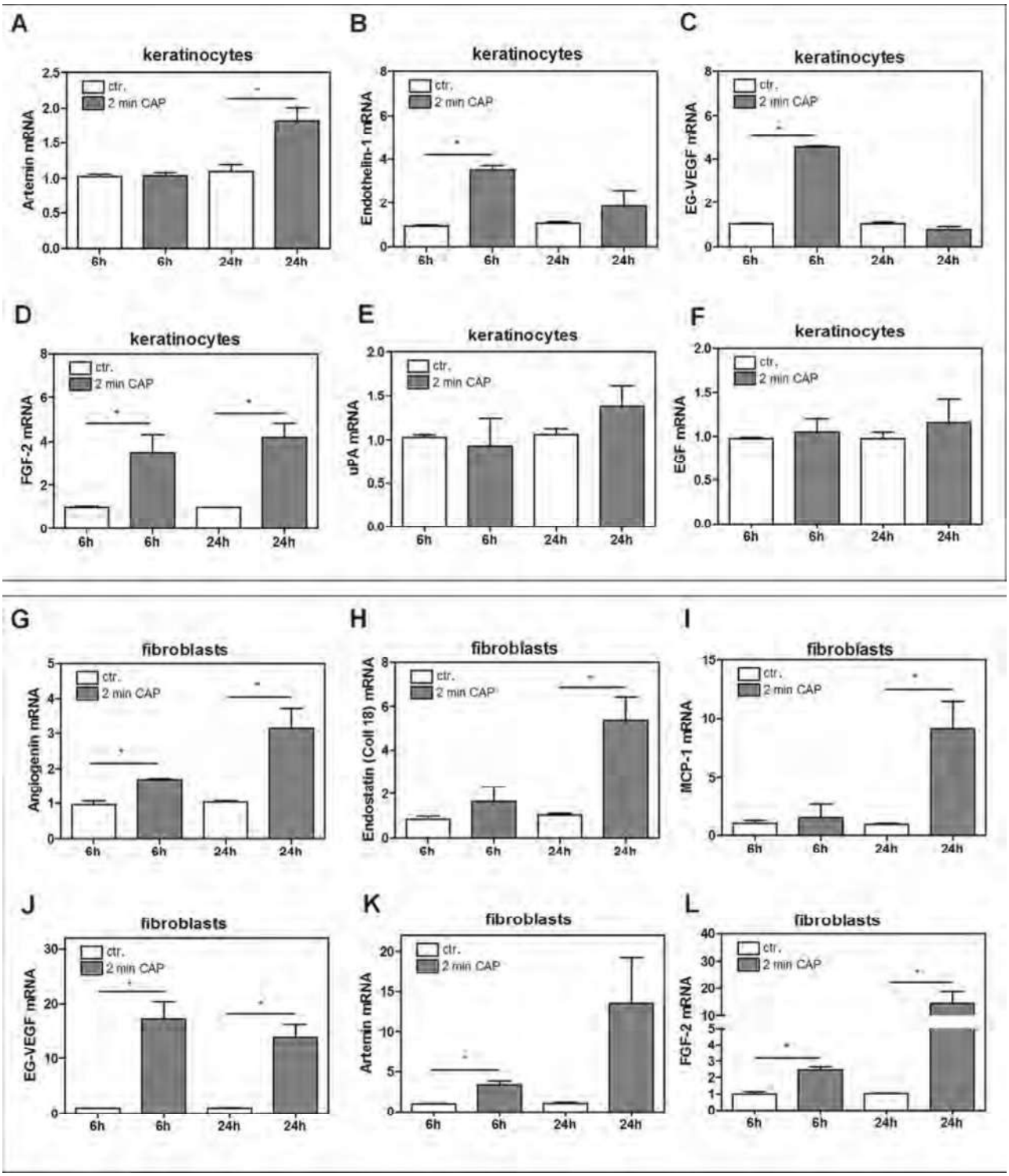

Fig. 3. mRNA expression of pro-angiogenic genes in keratinocytes, fibroblasts and HUVECs after direct CAP exposure.
mRNA expression of several pro-angiogenic and angiogenesis-related factors was determined via LightCycler® 1.2 technology (A–F) in keratinocytes, (G–L) in fibroblasts and (M–P) in HUVECs. mRNA was obtained from keratinocytes and fibroblasts 6 h and 24 h after CAP treatment for 2 min, whereas HUVECs were treated with CAP for only 30 s. *$p < 0.05$.

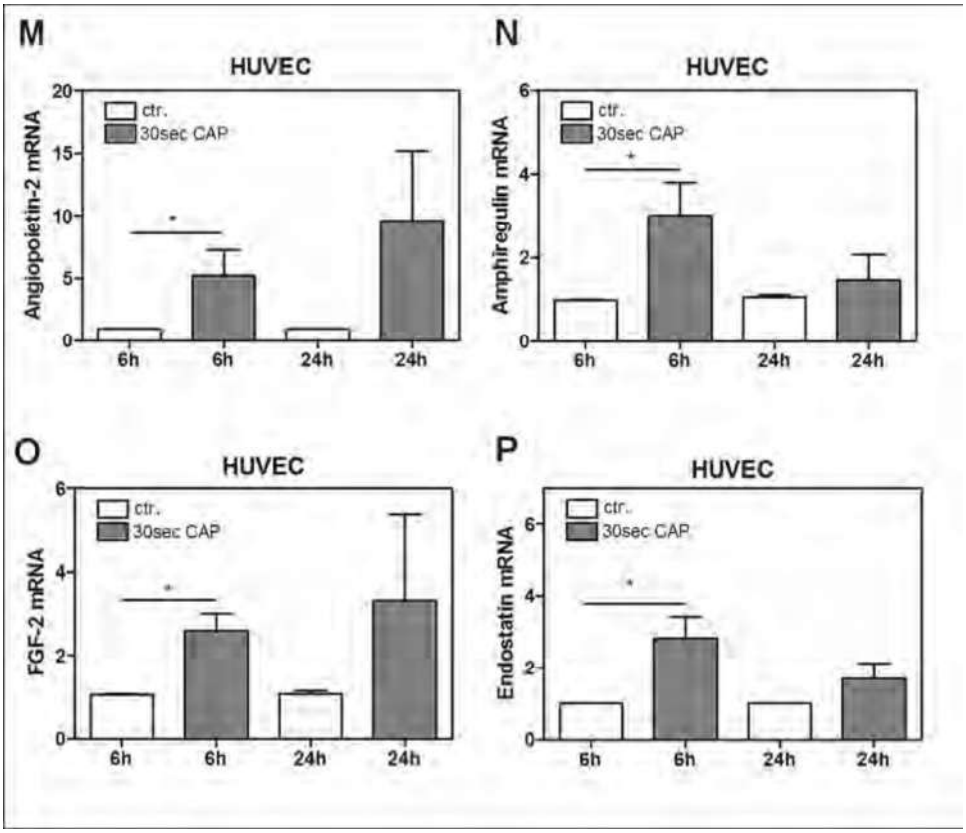

Fig. 3. (Continued)

3.2. CAP treatment promotes the induction of angiogenesis-related molecules in endothelial cells but also in fibroblasts and keratinocytes

Next, we were interested whether angiogenesis related molecules are affected in HUVECs after CAP treatment. Supernatants from HUVECs were collected 24 h after 30 s CAP treatment and protein expression of 55 angiogenesis-related proteins was analyzed using antibody array technology. We observed a significant upregulation (≥2-fold compared to the untreated control) of Angiopoietin-2 (2.58-fold ± 0.94), Angiostatin (PLG) (2.10-fold ± 0.18), Amphiregulin (2.99-fold ± 0.75), Endostatin (3.07-fold ± 0.92) and FGF-2 (2.99-fold ± 0.76) in HUVECs. Additionally we observed that the secretion of Artemin (2.68-fold ± 0.34), EGF (2.29-fold ± 0.24), EG-VEGF (2.20-fold ± 0.32), Endothelin-1 (2.21-fold ± 0.59), FGF-2 (2.78-fold ± 0.17), IL-8 (CXCL8) (5.32-fold ± 1.19) and uPA (2.16-fold ± 0.99) was elevated in keratinocytes after 2 min CAP treatment compared to the untreated control cells. Using the same treatment conditions, Angiogenin (3.15-fold ± 2.06), Endostatin (1.30-fold ± 0.03), MCP-1 (CCL2) (11.21-fold ± 1.89), MMP-9 (1.40-fold ± 0.07), TIMP-1 (1.39-fold ± 0.11), uPA (2.53-fold ± 1.55) and VEGF (3.00-fold ± 1.87) was induced in fibroblasts compared to the untreated control cells. These results suggest that the secretion of angiogenesis-related molecules is affected in endothelial cells as well as by fibroblasts and keratinocytes after the CAP treatment.

Array results were partially confirmed using ELISA technique 24 and 48 h after the CAP treatment and confirmed a significant induction of protein secretion of FGF-2 (after 24 h), Artemin (after 24 h) and EG-VEGF (after 24 h) by keratinocytes (Fig. 2A–C) and of FGF-2 (after 48 h) and MCP-1 (after 24 h) by fibroblasts after 2 min of CAP treatment (Fig. 2D, E). Additionally, we screened mRNA expression at different time points (6 and 24 h) after 2 min of CAP treatment in keratinocytes and fibroblasts, and after 30 s of CAP treatment in HUVECs. A significant mRNA induction was observed for Artemin (24 h), Endothelin-1 (6 h), EG-VEGF (6 h) and FGF-2 (6 h and 24 h) whereas no significant induction could be observed for uPA and EGF in keratinocytes at any of the analyzed time points after the CAP treatment (Fig. 3A–F). For fibroblasts we could confirm a significant mRNA induction of Angiogenin (6 h and 24 h), Endostatin (24 h), MCP-1 (24 h), EG-VEGF (6 h and 24 h), Artemin (6 h) and FGF-2 (6 h and 24 h) (Fig. 3G–L). For HUVECs we observed a significant mRNA induction of Angiopoietin-2 (6 h), Amphiregulin (6 h), FGF-2 (6 h) and Endostatin (6 h) after 30 s of CAP treatment (Fig. 3M–P).

In summary, these results suggest that CAP activates angiogenesis-related molecules in keratinocytes, fibroblasts and endothelial cells.

3.3. CAP is able to enhance tube formation by endothelial cells either directly or indirectly via paracrine mechanisms

As we observed an induction of FGF-2 after CAP treatment in endothelial cells (Fig. 3O) as well as in keratinocytes (Figs. 2 A, 3 D) and fibroblasts (Figs. 2 D, 3 L), we speculate that CAP-mediated induction of angiogenesis-related protein secretion is not limited to endothelial cells but also affects other cells, which in turn are able to affect angiogenesis in a paracrine mode.

We verified this hypothesis by performing tube formation assays using either endothelial cells directly treated with CAP for 30 s, 1 min and 2 min or by performing tube formation assays using

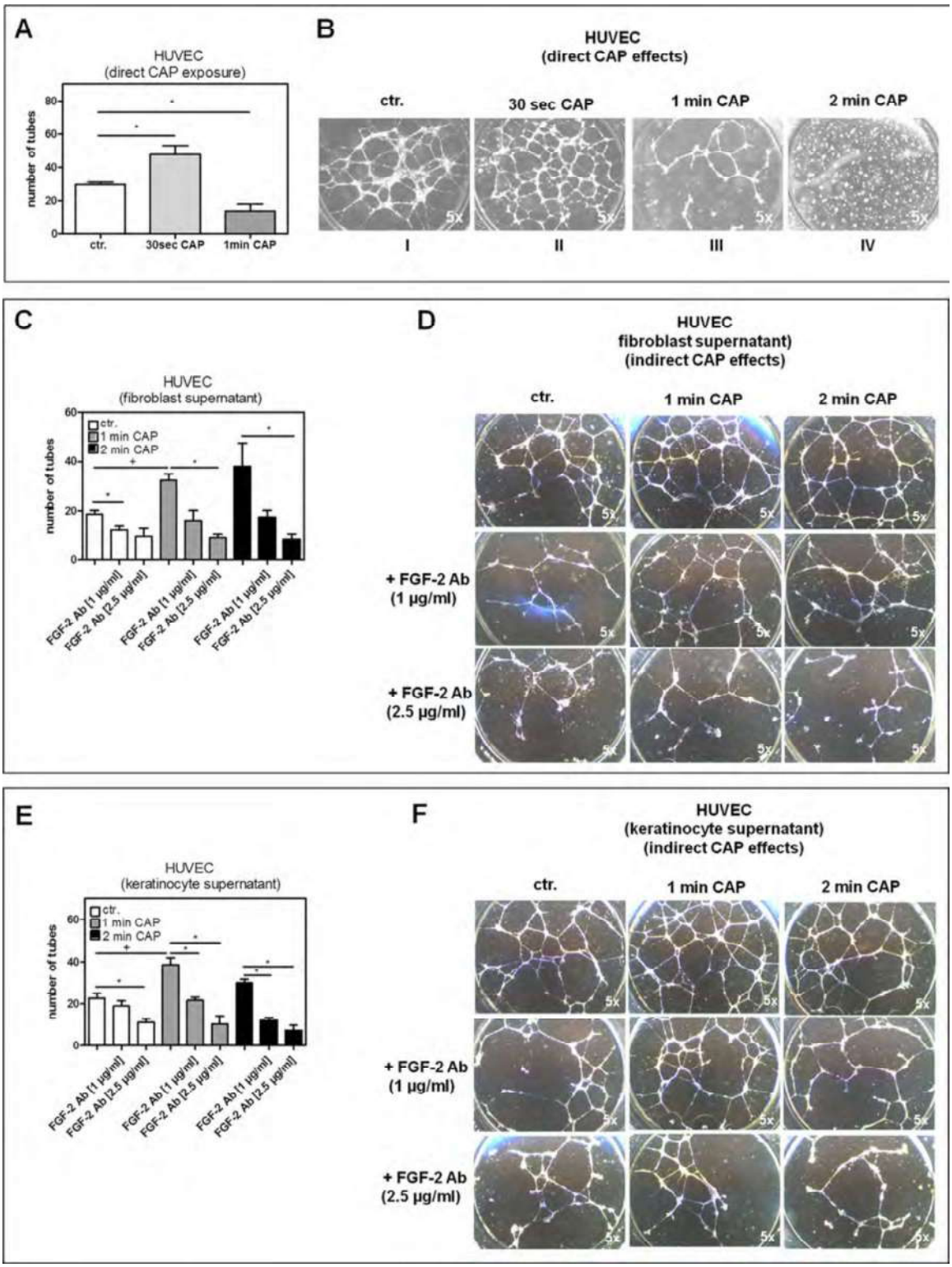

Fig. 4. Tube formations by HUVECs after direct and indirect CAP treatment.
(A) HUVEC cells were directly treated with CAP for 30 s, 1 min and 2 min and numbers of tubes were determined using the 15-well Angiogenesis µ-Slide System from Ibidi (Martinsried, Germany). (B) Pictures exemplarily show the tube formation by HUVECs after direct CAP treatment for 30 s, 1 min and 2 min. Tube formation was induced when cells were treated with CAP for 30 s compared to the untreated control (ctr.), whereas HUVECs form reduced numbers of tubes after 1 min CAP treatment and did not form any vascular sprouts after a 2 min exposure to CAP. Indirect CAP treatments with supernatants from CAP treated fibroblast (C, D) and keratinocytes (E, F) for 1 min and 2 min show an elevated tube formation compared to the corresponding untreated control (ctr.). Indirect CAP treatments with supernatants from CAP treated fibroblast and keratinocytes additionally supplemented with increasing amounts of FGF-2 neutralizing antibody show a reduced tube formation.
Tube formation was monitored by phase contrast microscopy after 24 h and recorded with a digital camera (Leica MC170HD). $^{*}p < 0.05$.

conditioned supernatants from fibroblasts and keratinocytes previously treated with CAP for 1 min and 2 min and further incubation of the cells for 24 h.

Interestingly, we observed that HUVECs, directly treated with CAP for 30 s, formed an increased tube network after 24 h compared to the untreated control cells (ctr.) (Fig. 4A and B I, II). Indeed, HUVECs treated with CAP for 1 min showed a reduced number of tubes compared to the control (ctr.) (Fig. 4A and B I, III) and further treatment (up to 2 min) even prohibited tube formation exemplarily shown in (Fig. 4B IV). HUVECs treated with supernatants obtained from fibroblasts (Fig. 4C and D) or keratinocytes (Fig. 4E and F) treated with CAP for 1 min or 2 min showed a pronounced tube formation compared to samples incubated with untreated control supernatants (ctr.). On the other hand, HUVECs treated with supernatants from CAP activated fibroblasts or keratinocytes additionally supplemented with increasing amounts of neutralizing FGF-2 antibody, significantly reduce tube formation.

These results show that CAP affects tube formation by HUVECs directly and via indirect mechanisms and suggest that FGF-2 secreted by fibroblasts and keratinocytes is an important pro-angiogenic factor and important to modulate the vasculature.

3.4. CAP induces angiogenesis-related receptor expression in endothelial cells via autocrine mechanisms

To get further insight into how CAP affects the mechanism of angiogenesis during wound healing, we focused on the receptor expression of EGF R, FGF R1-3, VEGF R1-3 and GFRA-3 (Artemin R) in HUVECs. We treated HUVECs indirectly with conditioned supernatants obtained from keratinocytes and fibroblasts after 2 min of CAP treatment and further incubation of cells up to 24 h. Additionally, HUVECs were treated directly with CAP for 30 s and further incubation up to 24 h.

Surprisingly, we observed no significant mRNA modulation of any of the analyzed receptors in HUVECs when they were treated indirectly with cell culture supernatants from keratinocytes (Fig. S.1) and fibroblasts (Fig. S.2). Interestingly, a significant mRNA induction was observed for FGF R1 (Fig. 5A), and VEGF R1 (Fig. 5B) in HUVECs directly treated with CAP for 30 s. ELISA experiments confirmed the induction of FGF R1 and VEGF R1 on protein level (Fig. 5C and D). The expressions of the other analyzed receptors were not significantly affected by the CAP treatment (data not shown).

3.5. Wound angiogenesis is induced after CAP treatment in vivo

We performed a murine wound healing model, where the wounds were treated with CAP for 2 min 10 days long, and tissue preparation was performed on day 15 after wounding [21]. Biopsies of the wounds treated with CAP and stained with anti-CD31 antibody showed an elevated vasculature 15 days after wounding compared to the placebo gas control (Fig. 6A, arrow-head). Likewise, CD31 and FGF-2 mRNA expression was induced in murine epidermal and dermal wound tissue treated with CAP compared to the placebo control (Fig. 6B and C). Future studies must be performed to determine further angiogenic-involved molecules affected by CAP treatments *in vivo*.

4. Discussion

Wound healing is a complicated and dynamic process and although a variety of therapeutic strategies have been adopted in clinical practice for chronic and complex non healing wounds,

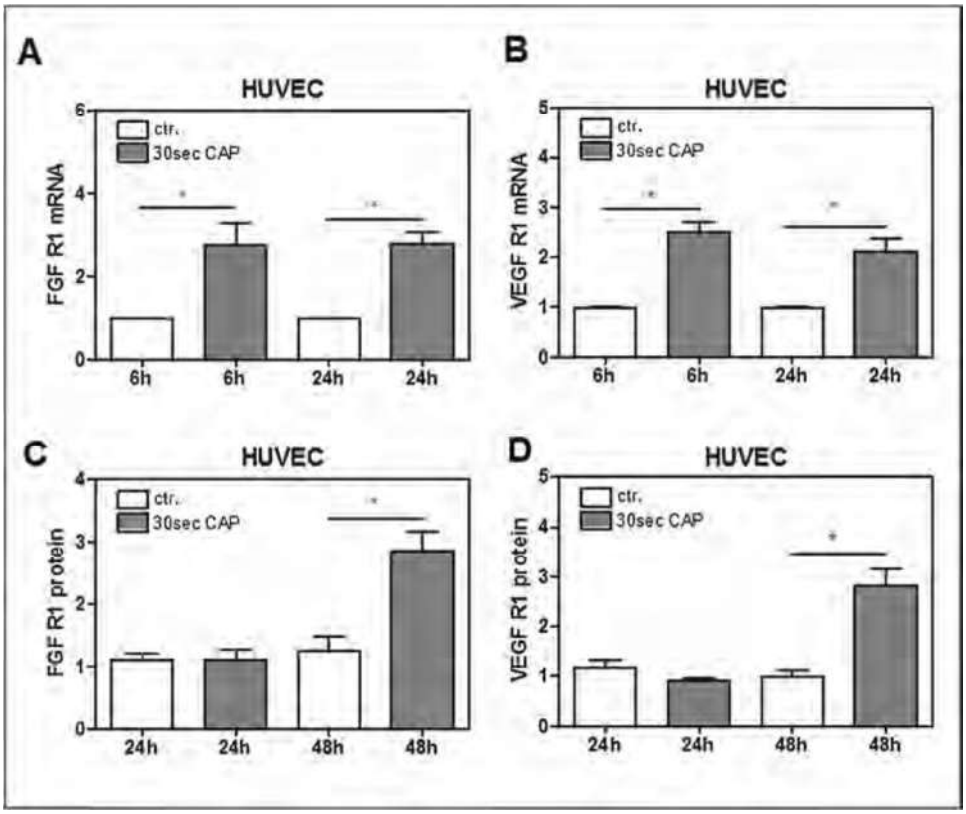

Fig. 5. mRNA and protein expression of angiogenic receptors in HUVECs after CAP treatment.
mRNA expression of angiogenesis-related receptors was determined via LightCycler® 1.2 technology. A significant induction was observed for FGF R1 (A, C) and for VEGF R1 (B, D) in HUVECs after direct CAP treatment for 30 s on mRNA and protein level. $^{*}p < 0.05$.

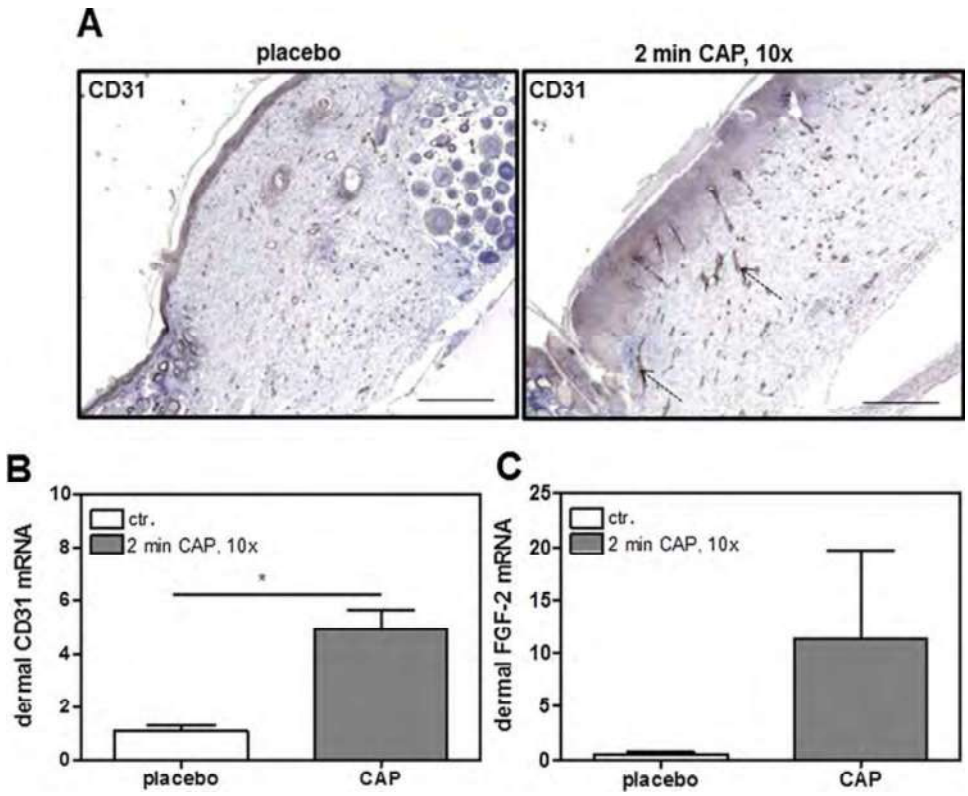

Fig. 6. Wound angiogenesis *in vivo* after CAP treatment.
(A) CD31 staining was performed on murine skin biopsies 15 days after wounding and 10 x CAP treatments for 2 min compared to the placebo control. mRNA expression of CD31 (B) and FGF-2 (C) was analyzed in dermal tissue after 10 × 2 min CAP treatment compared to the placebo control. *$p < 0.05$. Scale bar = 50 μm.

there is a need for alternative and effective therapies [25]. Cold atmospheric plasma (CAP) has shown promise for wound healing, although the underlying mechanisms are not fully understood. Different studies described a significant reduction of bacterial colonization in infected wounds without the negative effects of antibiotic treatment such as bacterial resistance [17,26,27]. Furthermore, in our own previous studies we could show that CAP is able to activate wound healing-related molecules in dermal fibroblasts and epidermal keratinocytes resulting in an accelerated wound healing [20,21]. In addition, a few studies already described a positive effect of CAP on wound angiogenesis but little has been reported on its cellular mode of action. CAP contains various components including electrons, charged ions, reactive oxygen species (ROS), reactive nitrogen species (RNS), and UV. Although it is unknown which components of the plasma "cocktail" are responsible for the observed effects of CAP, reports suggest that ROS and RNS promote vascularization through angiogenic growth factor mechanisms [12,14,28,29].

This study was designed to determine key players and molecular mechanisms which affect angiogenesis by the CAP treatment.

First, we tried to find out the optimal CAP treatment conditions for endothelial cells using the MicroPlaSter ß® device. In comparison to epidermal keratinocytes and dermal fibroblasts, which are relatively robust towards CAP treatments [20,21], our present results show that endothelial cells are more sensitive. We determined 30 s as optimal CAP treatment time for HUVECs using the MicroPlaSter ß® device. This result is in accordance with the observations published by Kalghatgi and co-workers showing that CAP treatments at short exposures (up to 30 s; 4 J/cm^2; using a DBD device) were relatively non-toxic to endothelial cells while longer exposures (60 s and higher or 8 J/cm^2) led to cell death. [11,30].

Based on the fact that *in vivo* endothelial cells are not directly affected by CAP treatments in the skin since CAP components do not penetrate deeply into the skin, we speculate that CAP might affect endothelial cells indirectly via paracrine mechanisms. Kalghatgi and co-authors published that CAP is able to affect endothelial cells directly and promotes the release of pro-angiogenic FGF-2 [11]. We could confirm that FGF-2 and other angiogenic-related factors like Angiopoietin-2 (pro-angiogenic factor), Amphiregulin (pro-angiogenic factor), and Endostatin (anti-angiogenic factor) are directly affected by CAP in endothelial cells (Fig. 3M–P). Further, *in vitro* experiments and tube formation assays using supernatants from CAP treated keratinocytes and fibroblasts show that paracrine mechanisms (communication between keratinocytes and endothelial cells and communication between fibroblasts and endothelial cells) are also involved and promote the activation of angiogenesis-related factors in endothelial cells. This data show for the first time that CAP affects vascularization via paracrine mechanisms. Thus, endothelial cells do not have to get in direct contact with the plasma gas but can be activated indirectly.

Interestingly, we observed that many angiogenesis-related factors are induced after CAP treatment, whereas the expression profile and kind of factors differ between fibroblasts, keratinocytes and endothelial cells. We suspect that the amount and induction of angiogenesis-related factor expression after the CAP treatment is cell specific and may depend on the sensitivity of the cell type towards CAP.

ROS possess the ability to regulate the formation of blood vessels at the wound site and the optimal perfusion of blood into the wound-healing area [31]. CAP treatment enables localized ROS/RNS application in precise doses. In light of this important role of reactive species in wound healing and the continued need for

therapeutic strategies to treat acute and especially chronic wounds, such as diabetic foot ulcers, venous and arterial leg ulcers and pressure ulcers in particular, the manipulation of ROS/RNS or other CAP components represents a promising avenue for improving wound-healing responses. Future studies must reveal which CAP components are finally responsible for the activation of the determined pro-angiogenic factor expression in the analyzed cells. With this knowledge we would be able to generate a probably even more effective plasma "cocktail" specific for the desired application.

Author contribution

S.A. designed the experiments and wrote the paper. S.A. performed the animal experiments and P.U. the *in vitro* experiments. S.A., S.K., M.B., and A-K.B. projected and designed the study and participated in data analysis and discussion.

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Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jdermsci.2017.11.008.

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