COMPARATIVE STUDY ASSESSING EFFECTS OF SONIC HEDGEHOG AND VEGF IN A HUMAN CO-CULTURE MODEL FOR BONE VASCULARISATION STRATEGIES

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Abstract

The morphogen sonic hedgehog (Shh) seems to mediate adult repair processes in bone regeneration and vascularisation. In this study we investigated the effects of Shh on co-cultures consisting of human primary osteoblasts and outgrowth endothelial cells in terms of angiogenic activation and vessel maturation in comparison to the treatment with the commonly used proangiogenic factor, VEGF. Both, stimulation with VEGF or Shh, leads to an increase in the formation of microvessel-like structures compared to untreated controls. In contrast to VEGF, proangiogenic effects by Shh could already be observed after 24 h of treatment. Nevertheless, after 14 days the angiogenic activity of OEC was comparable in VEGF- or Shh-treated co-cultures. Furthermore, Shh and VEGF resulted in different growth factor expression or release profiles. Compared to VEGF, Shh stimulates also the expression and secretion of angiopoietins which was detected as early as 24 h of treatment. Moreover, smooth muscle cell-related markers, such as α-smooth muscle actin, desmin and myocardin, as well as basement membrane components were clearly upregulated in response to Shh treatment compared to VEGF- or untreated controls. In terms of growth factors relevant for vessel stabilisation and maturation increased levels of PDGF-BB, angiopoietin-1 and TGF-β were observed in cell culture supernatants when treated with Shh. This was in accordance with higher levels of smooth muscle actin in Shh-treated samples indicating the potential of Shh to improve the angiogenic activity and vessel stabilisation of human tissue engineered constructs. Experiments using cyclopamine, a Shh pathway inhibitor, blocked the effects of Shh.

Keywords: bone repair, neovascularisation, signalling molecules, co-culture

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Introduction

Identification and profound knowledge of molecular signalling pathways which control angiogenesis and osteogenesis during bone regeneration might offer new therapeutical options in tissue engineering and regenerative medicine. Apart from the more commonly used strategies to improve the vascularisation of tissue engineered bone constructs which are widely based on the treatment with classical pro-angiogenic growth factors, such as VEGF, several studies have focused on the role of various morphogens or signalling factors originally guiding embryonic development (Pola *et al.*, 2001; Dufourcq *et al.*, 2002). One promising developmental signalling pathway with significant relevance for angiogenesis and osteogenesis is mediated by Sonic hedgehog (Shh). Hedgehog proteins are originally known as morphogens regulating epithelial-mesenchymal cell interactions which are essential for the development of the limb bud, bones, the nervous system or the lung (Johnson *et al.*, 1994; Ruiz i Altaba, 1994; Chiang *et al.*, 1996). Three vertebrate homologues of *Drosophila* Hh, namely sonic hedgehog, desert hedgehog and Indian hedgehog (Fietz *et al.*, 1994) are currently known. Recent studies suggested that sonic hedgehog might be also involved in postnatal vascularisation (Pola *et al.*, 2001; Straface *et al.*, 2008) and osteoblastic differentiation in adults (Nakamura *et al.*, 1997). On the molecular level hedgehog signalling is mediated by the interaction with its receptor patched1 (Ptch1) which leads to the activation of smoothened and finally results in the activation of the Gli transcription factors. Activated Gli accumulates in the nucleus and controls the transcription of hedgehog target genes, including Ptch1 and Gli themselves (Cohen, 2003; Nagase *et al.*, 2007).

Other approaches to enhance and to accelerate the vascularisation of tissue engineered constructs are based on various delivery strategies for pro-angiogenic cell types or prevascularisation strategies including vascular structures or endothelial cells within a bioengineered tissue (Rouwkema *et al.*, 2006; Rivron *et al.*, 2008). In this context several potential autologous cell sources for pro-angiogenic cell types such as cells from adipose tissue-derived stromal fraction (SVF) (Scherberich *et al.*, 2007; Muller *et al.*, 2009), endothelial progenitor cells from different sources such as the bone marrow (Quirici *et al.*, 2001), the cord blood (Murohara *et al.*, 2000; Schmidt *et*

al., 2004) or adult peripheral blood (Kalka *et al.*, 2000; Peichev *et al.*, 2000) are currently discussed. The mechanisms by which pro-angiogenic cells contribute to the neovascularisation process include paracrine mechanisms based on the production of angiogenic growth factors leading to the angiogenic activation and proliferation of endothelial cells resident in the tissue (Gulati *et al.*, 2003; Rehman *et al.*, 2003). On the other side progenitor or stem cell subpopulations contained in the heterogeneous pro-angiogenic cell fractions are supposed to support the neovascularisation through differentiation into mature and functional endothelial cells (Yoon *et al.*, 2005). While those proposed pro-angiogenic cell types differ strongly in their endothelial characteristics and methods of action, a series of pro-angiogenic cell types have proven their ability to contribute to the neovascularisation process *in vivo* (Melero-Martin *et al.*, 2007). In terms of a therapeutical application a detailed definition of the pro-angiogenic cell types as well as the understanding of mechanisms leading to the pro-angiogenic effects *in vivo* is necessary in order to choose the most suitable cell type depending on the clinical application.

One subpopulation with endothelial characteristics contained within endothelial progenitor cell cultures from the peripheral blood are so called outgrowth endothelial cells or endothelial cell colony-forming cells. The angiogenic potential of these outgrowth endothelial cells (OEC) *in vitro* has been documented in several previous studies (Sieminski *et al.*, 2005; Fuchs *et al.*, 2006b). Nevertheless, the formation of perfused vascular structures by OEC i*n vivo* seems to depend on the communication with other cell types, such as smooth muscle cells, mesenchymal stem cells or osteoblastic cells, which guarantees the strict control of the angiogenic process (Au *et al.*, 2008; Melero-Martin *et al.,* 2007; Fuchs *et al.,* 2009a). Besides this interest in co-cultures or co-implantations from a therapeutic point of view, co-cultures used as model systems may provide new insights into the molecular and cell biological mechanisms of repair processes and might be helpful in identifying targets for therapeutic intervention.

One option to accelerate and improve the bone repair process and in particular the formation of microvessel-like structures might be the treatment of tissue engineered constructs with growth factors or morphogens such as sonic hedgehog described in the previous sections. Although the overexpression of vascular endothelial growth factor (VEGF) leads to the induction of new vessels (Mandriota and Pepper, 1997; Pettersson *et al.*, 2000; Nagy *et al.*, 2002a; Nagy *et al.*, 2002b), these VEGF induced vessels lack pericytes, are unstable, perforated and tend to regress (Sundberg *et al.*, 2002). During vessel maturation, which is necessarily required for a stable and long lasting blood vessel system, several growth factors and signalling molecules orchestrate the individual steps of vessel assembly and recruitment of mural cells (Hellstrom *et al.*, 1999; Gaengel *et al.*, 2009). These processes are mediated by platelet derived growth factor (PDGF-BB) and its receptor-beta (PDGFR-β), as well as the angiopoietin/tie2 and transforming growth factor β (TGF-β) signalling systems (Hellstrom *et al.*, 2001; Gaengel *et al.*, 2009).

In a previous study a crucial function of Shh signalling pathway in promoting both angiogenic activation as well as osteogenic differentiation was demonstrated in a co-culture system consisting of primary osteoblasts (pOB) and outgrowth endothelial cells (OEC) (Dohle *et al.*, 2010). In this previous paper we already demonstrated that the angiogenic activation was at least partly mediated through the upregulation of VEGF and angiopoietins when compared to untreated co-cultures. By influencing both groups of angiogenic factors, VEGF and angiopoietins, Shh might also effect vessel maturation. As a consequence, the scope of the present study was to gain insight in Shh mediated effects on vessel stabilisation in comparison to the commonly used pro-angiogenic factor VEGF which is known to be associated with a leaky and immature vasculature.

Materials and Methods

Co-culture of outgrowth endothelial cells (OEC) and primary osteoblasts (pOB)

Outgrowth endothelial cells and primary osteoblasts were isolated and cultured as previously published (Hofmann *et al.*, 2003; Fuchs *et al.*, 2006a; Fuchs *et al.*, 2007). Co-cultures of OEC and pOB were seeded on Thermanox coverslips (12mm in diameter) (Fuchs *et al.*, 2007). Primary osteoblasts were seeded first at a density of 300,000 cells/well in a fibronectin-coated 24-well plate followed by seeding of 200,000 OEC/well 24 h later. Cells were co-cultivated in EGM-2 with supplements from the kit including low VEGF concentrations (2 ng/ml, according to the manufacturer), 5% FCS and 1% P/S for different time periods. For each co-culture experiment at least three different donors were used.

Stimulation of co-cultures with Sonic hedgehog or VEGF

Co-cultures were seeded as previously described on Thermanox coverslips in 24-well plates cultured in EGM-2 with supplements from the kit, 5% FCS and 1% Penicillin/Streptomycin. After 1 week of co-cultivation, cells were treated either with 5 µg/ml recombinant human sonic hedgehog (Shh) (R&D Systems, Wiesbaden, Germany) or 50 ng/ml (final concentration) VEGF in EBM-2 with supplements from the kit, 5% FCS and 1% P/S for 24 h and 14 days. Stimulated co-cultures were further processed for immunofluorescent staining, gene expression analysis and protein analysis. In addition, OEC and pOB monocultures were stimulated with recombinant Shh, serving as controls. In control studies co-cultures were treated with 5 µg/ml Shh plus the Sonic Hedgehog inhibitor Cyclopamine (Merck, Darmstadt, Germany) at different concentrations (5 µM, 10 µM, 20 µM) in EGM-2 with supplements from the kit, 5% FCS and 1% P/S for 14 days. Stimulated co-cultures were further processed for gene expression and protein expression analyses as described in the corresponding sections.

Cryostat sectioning

Cell layers of co-cultures consisting of OEC and pOB were snap frozen in liquid nitrogen and sectioned at a thickness of 10 µm using a cryostat (Leica Microsystems, Wetzlar, Germany). Samples were stored at -20°C until use for immunohistochemical analysis. For immunofluorescent staining the slices were first thawed at room temperature before starting with the staining procedure as described in the following section.

Immunofluorescent staining

For immunofluorescent staining cells were fixed with 3.7% paraformaldehyde (PFA) (Merck, Darmstadt, Germany), washed three times with PBS and then permeabilised for 5 min using 0.1% Triton-X in PBS. Cells were washed again with PBS before being incubated with different primary antibodies diluted in 1% bovine serum albumin (BSA) /PBS for 45 min at room temperature: CD31 (diluted 1:50; Dako, Hamburg, Germany); α-smooth muscle actin (diluted 1:100; Dako); desmin (diluted 1:100; Dako); laminin (diluted 1:200; Sigma-Aldrich, St. Louis, MO, USA); collagen IV (diluted 1:100; Sigma-Aldrich); von Willebrand factor (vWF) (diluted 1:8000; Dako). After washing 3 times with PBS, cells were incubated with fluorescently labelled secondary antibodies (Alexa; Molecular Probes, MoBiTec, Göttingen, Germany) diluted 1:1000 in 1% BSA in PBS for 45 min in darkness at room temperature. Finally, cell nuclei were counterstained with 1 µg/ml Hoechst and cells were mounted with Gelmount (Biomeda, Foster City, CA, USA). The stained samples or frozen sections were examined using a confocal laser scanning microscope (LeicaTCS-NT) (Leica Microsystems).

Quantitative real-time polymerase chain reaction (qreal-time PCR)

RNA isolation was performed using RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). One µg of extracted RNA was used to transcribe into complementary DNA (cDNA) according to a standard protocol using Omniscript Reverse Transcription Kit (Qiagen). Quantitative real-time PCR, enabling the quantification of relative gene expression, was performed using SYBR green DNA binding fluorescent dye. 12.5 µL of QuantiTect™ SYBR® Green PCR Master Mix, 2.5 µL of QuantiTect™ SYBR® Green primer assay (patched1, angiopoietin-1, angiopoietin-2, collagenIV, laminin, α-smooth muscle actin, desmin, myocardin, PDGF-BB, TGF-β; all provided by Qiagen, 6 µL of RNase free water and 4 µL of cDNA (1 ng/µL) were used for one reaction. Quantitative real-time PCR was performed in triplicates with the following cycler program: 95°C 15 min, denaturation step: 94°C 15 s, annealing step: 55°C 30 s, elongation step: 72°C 35 s; dissociation: 95°C 15 s, 60°C 1min, 95°C 15 s, 40 cycles were performed in total. Glycerin-Aldehyde-3-phosphate (GAPDH) or ribosomal protein 13A (RPL13A) was taken as an endogenous standard and relative gene expression was determined using the ΔΔCt method. Gene expression was compared by setting control cultures to 1 (reference value) as indicated in the relevant figures.

Enzyme-linked immunosorbent assay (ELISA)

Culture supernatants from different treated cells were collected and the concentration of different growth factors was measured using ELISA DuoSets® (R&D Systems). ELISA was performed according to the manufacturer's protocol in triplicate. A streptavidin-HRP (horseradish-peroxidase) colorimetric reaction was used to visualise protein concentrations. The optical density of each well was measured using a microplate reader (GENios plus, TECAN, Crailsheim, Germany) and a wavelength of 450 nm. Results are depicted in ratio to the control (control = 100%) or shown additionally as absolute values as indicated in the relevant figures.

SDS-page and Western Blot

For cell protein extraction cells were trypsinised, centrifuged and finally lysed with 0.1% Triton-X in 0.1 M Tris buffer ph 7.2. Cell lysates were mixed for 45 min at 4°C, centrifuged and supernatants were transferred to new tubes and stored at -20°C until use. A BCA (bicinchoninic acid) protein Assay Reagent Kit was used to determine the protein concentration according to the manufacturer's protocol (Pierce, Thermo Fischer, Bonn, Germany). Protein lysates were separated according to their molecular weight using SDS polyacrylamide gel electrophoresis (SDS-Page). Approximately 20 µg protein was mixed with RotiLoad-1 loading buffer (1:4) and incubated at 95°C for 5 min for protein denaturation before loading the samples into the wells of the stacking gel. Separation of proteins was performed at 25 mA in SDS-running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS). Separated proteins were transferred from the gel onto a nitrocellulose membrane to make the proteins accessible for the antibody detection using a mini transfer chamber filled with SDS transfer buffer (25 mM Tris-HCl, pH 8.0, 100 mM glycine, 25% methanol) for 1 h at 350 mA. The membrane was blocked in 5% milk powder containing 0.2% Tween PBS (blocking solution) for 1 h at room temperature and subsequently incubated with the primary antibody α-smooth muscle actin (Dako) diluted 1:100 in blocking solution overnight at 4°C. After washing 3 times with PBS 0.2% Tween for 5 min, each membrane was incubated with the HRP-conjugated secondary antibody diluted in blocking solution for 2 h at room temperature. Antibody was detected using enhanced chemiluminescent (ECL) detection reagents. Then the membrane was stripped with stripping buffer (100 mM Glycine-HCl pH 2.8) and incubated with an antibody against ERK-2 to assess equal protein loading per lane.

Image quantification

Microscopic images of immunofluorescence-stained co-cultures in response to different treatments were analysed using ImageJ 1.43 as previously published (Fuchs *et al.*, 2009b). Statistical analyses was performed with MS-Excel (Microsoft Office; Microsoft, Munich, Germany) and

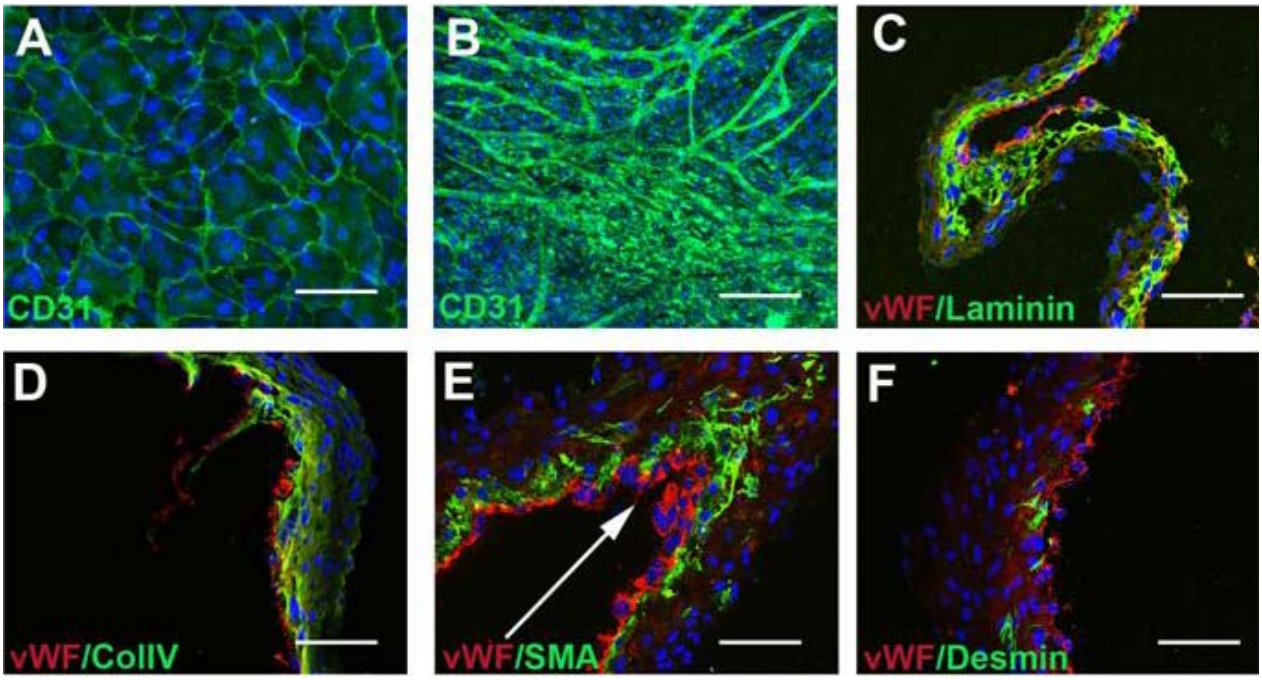

Fig. 1. Vessel maturation and stabilisation in untreated co-cultures. OEC and primary osteoblasts were co-cultivated for 4 weeks and stained for the endothelial marker CD31 (**B**; n=10). In contrast to OEC in monoculture **(A)**, OEC organize into microvessel-like structures after 4 weeks of co-cultivation with pOB, reminiscent of a microvasculature **(B)**. Cryostat sections of cultures co-cultivated for 4 weeks reveal the existence of the basement membrane molecules laminin **(C)** and collagen-IV **(D)** as well as the presence of stabilising **a**-smooth muscle actin- and desmin-positive cells closely associated with vascular structures, as depicted in **E** and **F**; n=3. Cell nuclei were counterstained with Hoechst. Scale bars: **A**, **C**-**F** = 75μm; **B** = 150μm.

statistical significance was evaluated using the paired Student's *t*-test (*p*-value **p*<0.05 and ***p*<0.01).

Statistical analyses

Data are represented as mean values ± standard deviation of the mean. Statistical significance was assessed using the paired students *t*-test (*p*-value **p*<0.05 and ***p*<0.01) and MS-Excel (Microsoft).

Results

Morphological assessment of untreated co-cultures in terms of factors involved in vessel maturation and stabilisation

Previous studies already documented the angiogenic potential of OEC co-cultured with primary osteoblasts. In contrast to OEC in monoculture (Fig. 1A), OEC form considerable angiogenic structures when co-cultivated with primary osteoblasts which increase during the course of co-cultivation as depicted in Fig. 1B (n=10). These co-cultures were not treated with additional external factors and served as controls for the following experiments. Functional and stable vessels necessarily require mechanical support through the formation of a basement membrane and the recruitment of stabilising mural cells including pericytes and smooth muscle cells. Immunofluorescent staining for the basement membrane proteins laminin and collagen-IV was performed to detect these proteins in untreated co-cultures of OEC and pOB after 4 weeks of cultivation (Fig. 1C/D; n=3). Von Willebrand factor-positive cells decorating the endothelial cells within the co-culture are clearly surrounded by the basement membrane components laminin and collagen IV. To investigate whether stabilising smooth muscle cells are present during the formation of microvessel-like structures in untreated co-cultures of OEC and pOB, cryostat-sections of co-cultures cultivated for 4 weeks were stained immunohistochemically for the smooth muscle cell-associated markers, α-smooth muscle actin and desmin (Fig. 1E/F). Endothelial cells in microvessel-like structures are closely associated or co-localised with α-smooth muscle actin- and desmin-positive (Fig. 1E/F) cells.

Differences in the angiogenic activation dependent on sonic hedgehog or vascular endothelial growth factor stimulation in co-cultures of pOB and OEC

To compare the effects of Shh and VEGF on the angiogenic activation of OEC, co-cultures were treated with individual factors for different time points to assess short and longer time treatment effects. Stimulation with both factors, vascular endothelial growth factor (VEGF) and sonic hedgehog (Shh), leads to an increase in the formation of microvessel-like structures compared to untreated controls after 14 days of stimulation (Fig. 2A, n=6). In contrast to VEGF treatment, effects by Shh could already be observed after 24 h as indicated by the formation of tube-like structures and interconnected networks (Fig. 2A). Addition of VEGF could not induce the formation of angiogenic structures at the earlier time point of investigation after 24

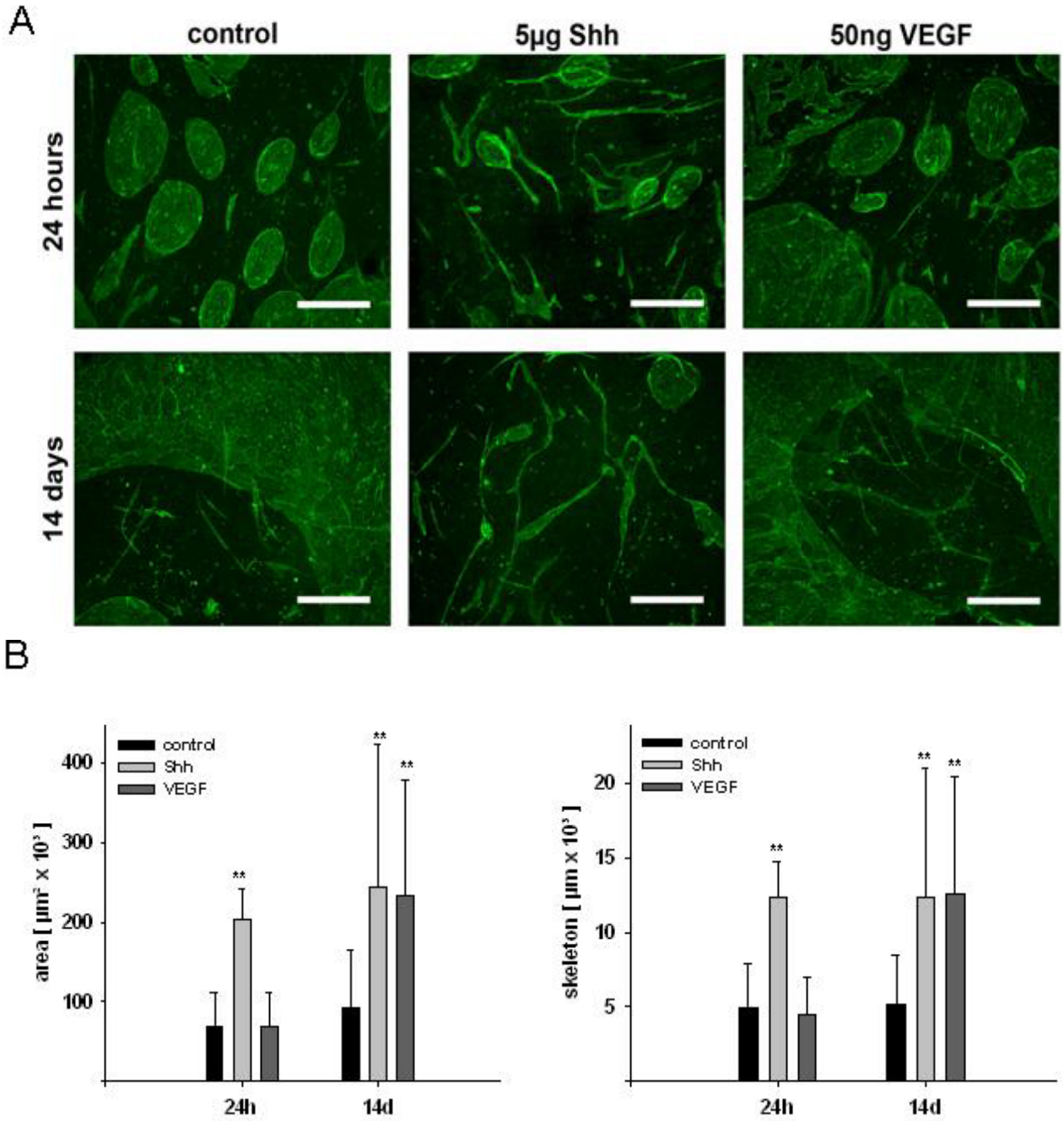

Fig. 2. Effects of sonic hedgehog treatment and vascular endothelial growth factor stimulation on co-cultures of OEC and pOB. Co-cultures of pOB and OEC were treated with 5 mg/ml Shh or 50 ng/ml VEGF for 24 h and 14 days and stained for the endothelial marker CD31 (**A**; n=6). Angiogenic structures were quantified by comparing total area of angiogenic structures and skeleton length of Shh- and VEGF-treated co-cultures after 24 h and 14 days of stimulation (**B**; n=4 different donors, 12 pictures in total were analysed for treatment or time point, respectively) [*p<0.05, **p<0.03]. Scale bars = 300µm.

h in the co-culture system. After 14 days of treatment the amount of microvessel-like structures was comparable in VEGF and in Shh stimulated co-cultures. These morphological findings were confirmed by quantitative analyses of angiogenic structures in Shh or VEGF treated co-cultures (Fig. 2B; n=4). The total area as well as the total skeleton length of microvessel-like structures, were significantly increased after 24 h of stimulation with Shh compared to VEGF treatment or untreated controls. In contrast, after 14 days of stimulation no significant differences in the area of angiogenic structures and total skeleton length could be observed between VEGF- and Shh-treated cultures (Fig. 2B).

Effects of sonic hedgehog and VEGF treatment on angiopoietins

VEGF and angiopoietins are growth factors controlling the angiogenic process. Therefore, the relative gene expression of the angiogenic factors, angiopoietin-1 and angiopoietin-2 was assessed in response to VEGF and Shh treatment. In comparison to VEGF and untreated controls, Shh treatment resulted in an upregulation of angiopoietin-

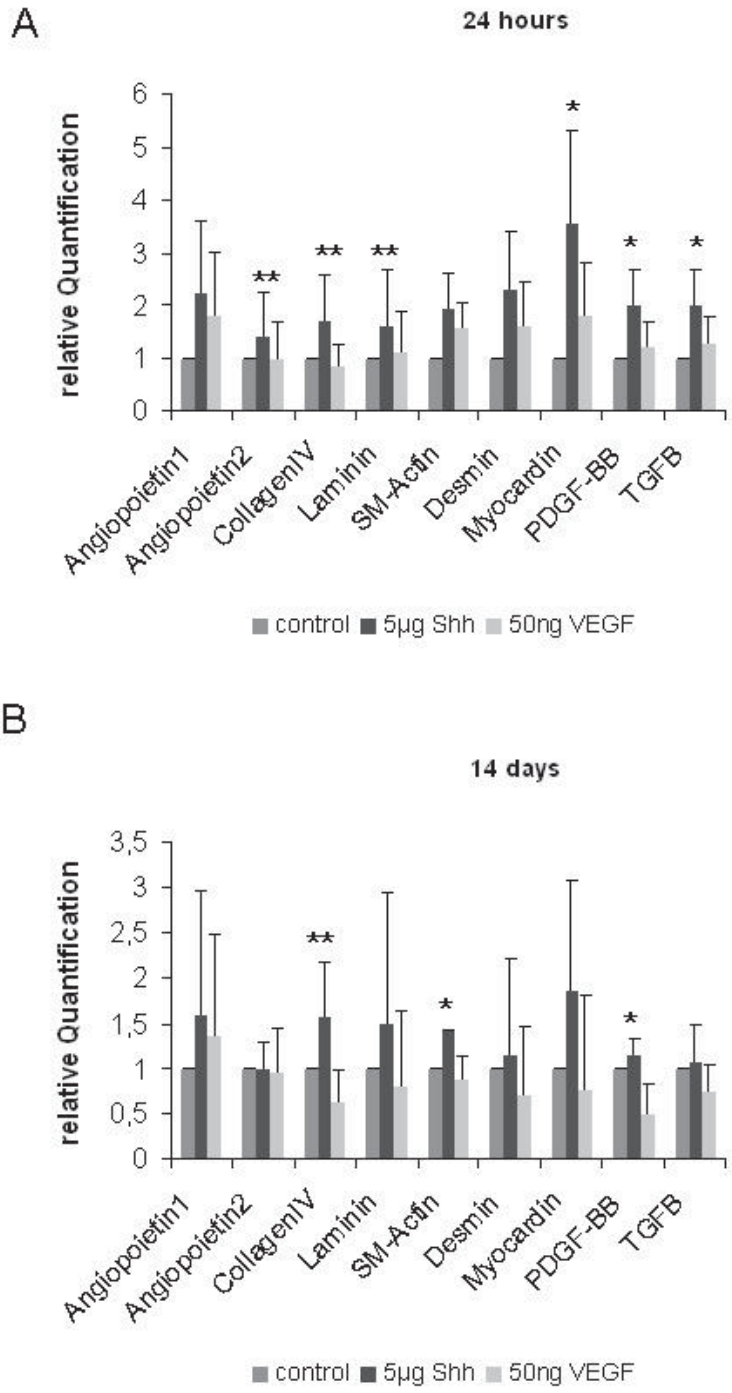

Fig. 3. Effects of sonic hedgehog and vascular endothelial growth factor on the expression of genes related to angiogenic activation and vessel stabilisation. Co-cultures of pOB and OEC were treated with 5 µg/ml Shh and 50 ng/ml VEGF for 24 h and 14 days. Quantitative real time PCR analysing relative gene expression of angiopoietin-1, angiopoietin-2, collagen-IV, laminin, α-smooth muscle actin, desmin, myocardin, platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β) was performed in response to Shh and VEGF treatment compared to control co-cultures. GAPDH was taken as an endogenous standard, and control co-cultures were set to 1 [**p*<0.05, ***p*<0.03] n=5.

1 after 24 h and 14 days of Shh stimulation (Fig. 3A,B; n=5). In addition, angiopoietin-2 was significantly upregulated in response to 24 h treatment with Shh (Fig. 3A, n=5), whereas after 14 days of stimulation no differences in gene expression of angiopoietin-2 could be observed (Fig. 3B; n=5).

We also measured the concentrations of angiopoietin-1 and angiopoietin-2 in the cell culture supernatants in response to Shh and VEGF treatment using an enzyme linked immunosorbent assay (Fig. 4, n=3). Angiopoietin-1 concentration was significantly increased in the supernatants of Shh-treated co-cultures after 24 h and after 14 days of stimulation when compared to VEGF-treated co-cultures and untreated controls (Fig. 4B). Furthermore, Shh treatment of co-cultures resulted in a significant increase in the release of angiopoietin-2 in the co-culture supernatants compared to respective controls at both time points of investigation (Fig. 4D). Nevertheless, the total amount of angiopoietin-2 determined from the supernatants is significantly higher in VEGF-treated compared to Shh-treated co-cultures when treated for 24 h or 14 days (Fig. 4D).

Effects of Shh and VEGF treatment on factors involved in vessel maturation and stabilisation

In addition to the angiopoietins we assessed several additional structural components, growth factor or signalling molecules involved in vessel assembly and stabilisation. Quantitative real time PCR was performed to examine gene expression levels of the basement

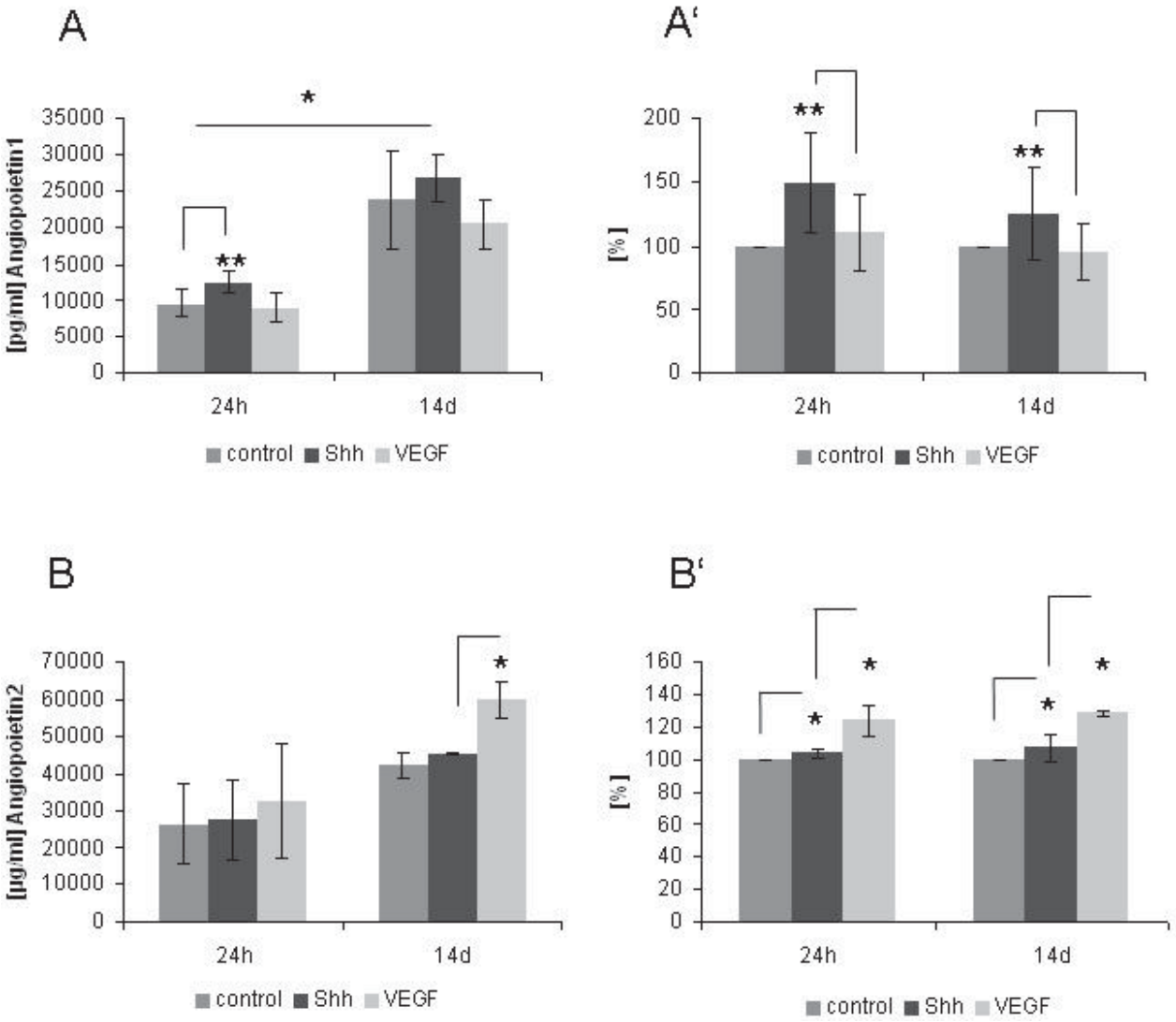

Fig. 4. Effects of Shh and VEGF treatment on the release of angiopoietin-1 and angiopoietin-2 in co-cultures of pOB and OEC. The concentration of angiopoietin-1 **(A,B)** and angiopoietin-2 **(C,D)** in the supernatants of co-cultures after 24 h and after 14 days of stimulation was measured using an enzyme-linked-immunosorbent-assay. Results are depicted as ratios with respect to the corresponding controls (control = 100%; **B,D**) and additionally shown as absolute values **(A,C)** [**p*<0.05, ***p*<0.03] n=3.

membrane molecules laminin and collagen IV, for the smooth muscle cell/pericyte associated markers, α-smooth muscle actin, desmin, and myocardin, as well as for growth factors such as platelet derived growth factor BB and TGF-β (Fig. 3A/B; n=5) in response to Shh or VEGF treatment. Compared to VEGF treatment and untreated controls Shh leads to an upregulation of all tested factors at both time points of investigation. A significant upregulation could be detected for collagen, laminin, myocardin, PDGF-BB and TGF-β after 24 h of Shh stimulation (Fig. 3A). After 14 days only the upregulation of collagen IV, α-smooth muscle actin and PDGF-BB was found to be statistically significant (Fig. 3B).

In addition to the real time PCR experiments we assessed using an enzyme linked immunosorbent assay the levels of PDGF-BB in the supernatants of co-cultures as a growth factor associated with smooth muscle cell differentiation and vessel stabilisation by mural cells. In accordance with the increased expression of PDGF-BB at the mRNA level, a significant increase in PDGF-BB levels in the supernatants of Shh-treated OEC monocultures was observed already after 24 h in response to Shh treatment (Fig. 5A, n=3). Inhibition of the Shh pathway using the Shh inhibitor cyclopamine in different concentrations revealed a dose-dependent downregulation of PDGF-BB (Fig. 5B) in quantitative real time PCR after 14 days. The positive effect of Shh on the PDGF-BB expression in the co-culture was reduced by the inhibitor cyclopamine in close correlation with its concentration, although these effects were not statistically significant. Nevertheless, these blocking experiments further confirmed a correlation of Shh and several factors involved in vessel stabilisation. In addition to PDGF-BB also TGF-β and angiopoietin-1 expression was tentatively reduced in response to the Shh blocking agent Cyclopamine (Fig. 5B, n=6) in a concentration dependent matter.

The effect of Shh in comparison to VEGF on vessel stabilisation was further analysed at the protein level. Using

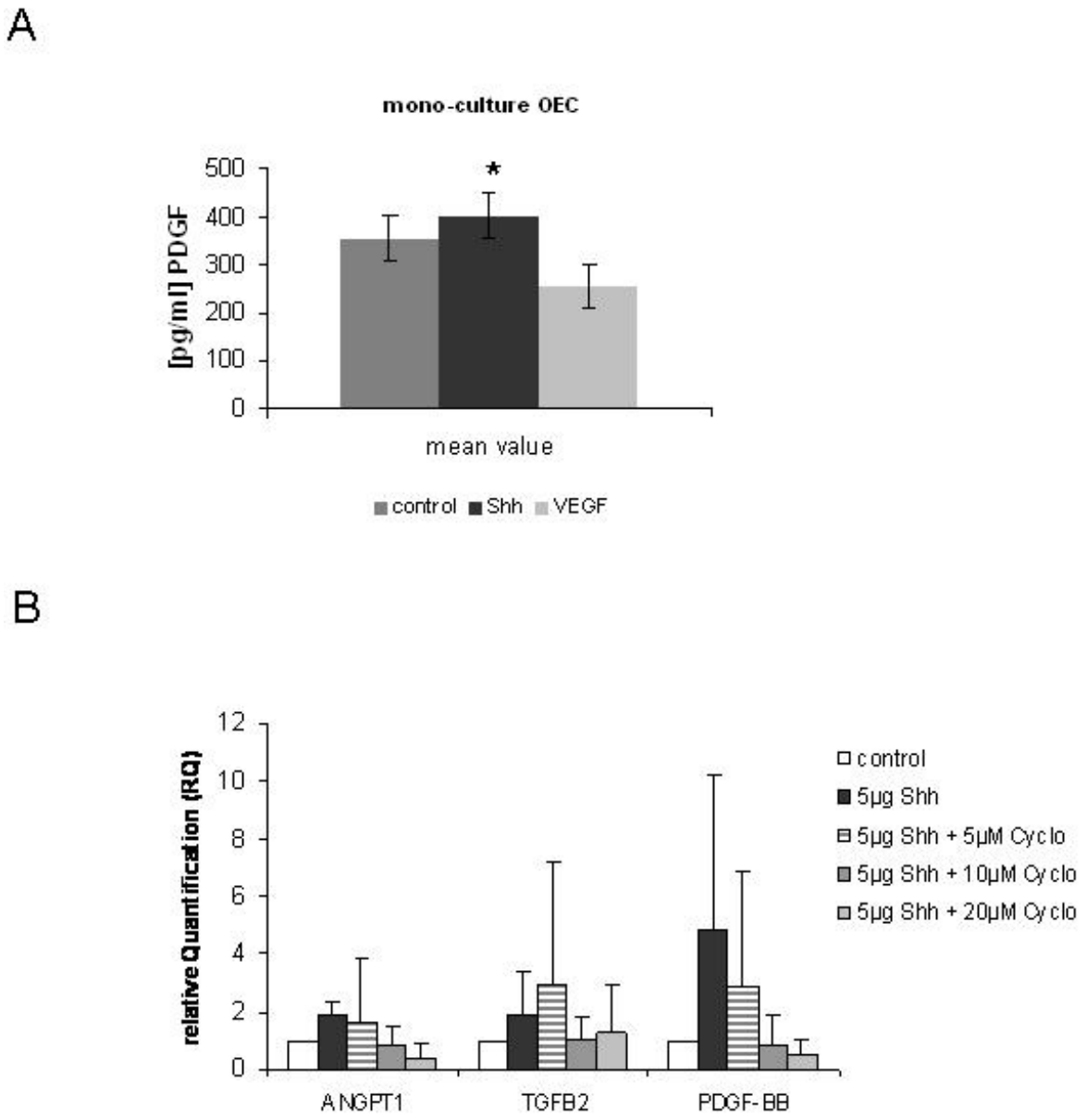

Fig. 5. Effects of Shh on PDGF-BB, angiopoietin-1 and TGF-β pathway activation. Concentration of PDGF-BB in supernatants of Shh- and VEGF-stimulated OEC monocultures **(A)**. After OEC monocultures were treated for 24 h with 5 µg/ml Shh or 50 ng/ml VEGF, PDGF-BB concentration was measured in the supernatants of the different treated cultures using an enzyme linked immunosorbent assay (ELISA). PDGF-BB protein concentration is depicted as absolute concentration. Inhibition of Shh signalling pathway demonstrates the dependency between Shh and angiopoietin-1, PDGF-BB and TGF-β pathway activation **(B)**. Co-cultures of OEC and pOB were treated either with 5 µg/ml Shh or simultaneously with 5 µg/ml Shh and the Shh inhibitor cyclopamine (Cyclo) in different concentrations (5 µM-20 µM) for 14 days. Quantitative real time PCR analysing relative gene expression of angiopoietin-1, PDGF-BB and TGF-β was performed and compared to control co-cultures. GAPDH was taken as an endogenous standard and control co-cultures were set to 1 [*p<0.05, **p<0.03] n=6.

SDS-Page and Western blotting the protein amount of α-smooth muscle actin in co-culture cell lysates was assessed in response to the different treatments and standardised to an internal control (Fig. 6A, n=4). These experiments revealed that the protein amount of α-smooth muscle actin increased during the course of co-cultivation from 24 h to 14 days. Nevertheless, the highest α-smooth muscle actin protein content was detected in Shh-stimulated co-cultures after 14 days of treatment (Fig. 6B), although this was not statistically significant due to the donor variation. The correlation of Shh and smooth muscle actin as indicator for vessel stabilisation was further assessed in blocking experiments using the Shh inhibitor cyclopamine. Inhibition of the Shh signalling pathway with 10 µM cyclopamine in the presence of Shh revealed a significant decrease of α-smooth muscle actin expression on the mRNA level evaluated by quantitative real time PCR (Fig. 6C, n=6). Similar effects in these blocking experiments were also observed on the protein level analysed by SDS-Page and Western blotting (Fig. 6D, n=6). On both levels of investigation, gene and protein expression, the highest α-smooth muscle actin levels could be detected in Shh

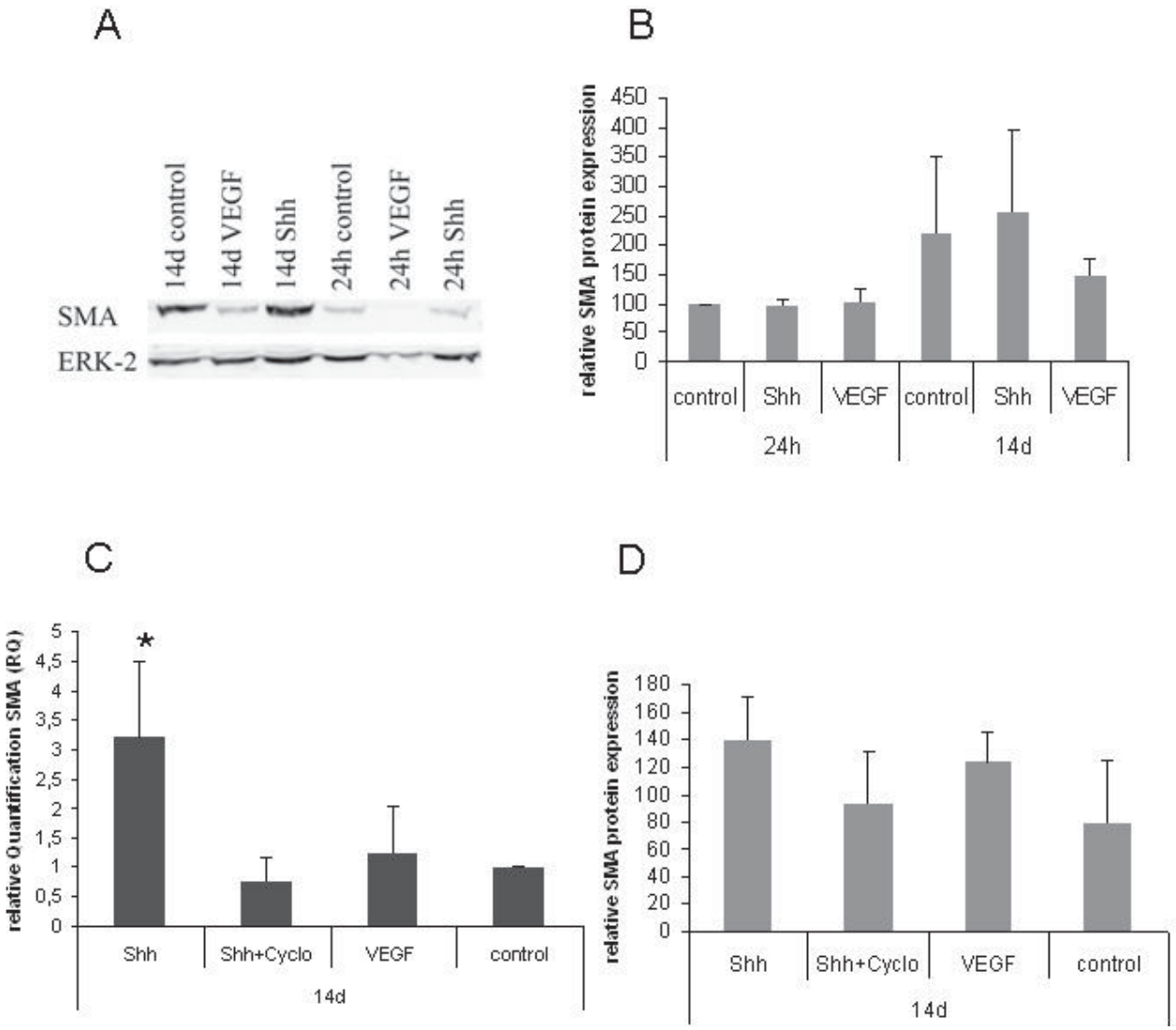

Fig. 6. Effects of Shh and VEGF on the smooth muscle cell related marker α-smooth muscle actin. The amount of α-smooth muscle actin in cell culture lysates of Shh- and VEGF-treated co-cultures after 24 h and 14 days of stimulation was compared using SDS-Page and Western blot **(A/B)**. Control co-cultures cultivated for 24 h were set to 100. n=4. After treatment of co-cultures with 5 µg/ml Shh and 50 ng/ml VEGF as well as inhibiting the Shh pathway by treating co-cultures simultaneously with 5 µg/ml Shh and 10 µM cyclopamine (Cyclo), alpha smooth muscle actin expression was analysed on the mRNA level using quantitative real time PCR **(C)** as well as on the protein level using SDS-Page and Western blot **(D)** [*p<0.05, **p<0.03] n=6.

stimulated co-cultures, whereas blocking of Shh by 10 µM cyclopamine (Fig. 6C/D) leads to a reduction of smooth muscle actin.

Discussion

Recent findings suggesting a significant role of the morphogen sonic hedgehog in bone regeneration and vascularisation have raised the question of whether Shh might be suitable to enhance bone repair processes in adults. Former results from our group have already shown that blocking of the sonic hedgehog pathway inhibits the formation of OEC derived angiogenic structures induced by the co-culture process. On the other hand the angiogenic activity of OEC in the co-culture system is enhanced in response to Shh treatment through the upregulation of pro-angiogenic factors, like VEGF or angiopoietins (Dohle *et al*., 2010). We also documented in this previous study that Shh leads simultaneously to the improved osteogenic differentiation of co-cultures. Apart from this positive effect of Shh on both elemental processes in bone regeneration, namely angiogenesis and osteogenesis, Shh might offer additional advantages in comparison to VEGF which is commonly used as a pro-angiogenic agent in the field of bone tissue engineering (Elcin *et al.*, 2001; Sun *et al.*, 2010). The present study demonstrates that in contrast to VEGF Shh supports several factors involved in vessel maturation and stabilisation. Absent vessel maturation and stabilisation are up to now the major problems associated with VEGF-based strategies to enhance the vascularisation process. In response to Shh treatment we observed an upregulation of angiopoietin-1, PDGF and TGF, accompanied with an increase of smooth muscle actin on

the protein and gene expression level. These positive effects of Shh on growth factors and markers involved in vessel maturation were blocked in the presence of the Shh pathway inhibitor cyclopamine.

Although it is generally accepted that ectopic transient VEGF expression leads to the induction of new vessels, these VEGF-induced vessels are very unstable because they lack pericytes (Sundberg *et al*., 2002). In order to generate a stable and functional and in particular long-lasting vasculature, newly formed vessels need to be stabilised through the recruitment of mural cells, including pericytes and vascular smooth muscle cells (Lee *et al*., 1997; Carmeliet, 2005).

In the present study treatment of co-cultures with Shh compared to treatment with the commonly used pro-angiogenic factor VEGF resulted in a significantly enhanced angiogenic activation of the OEC, which was already observed after 24 h of treatment. Nevertheless, in experiments using longer periods of treatment (14 days) the angiogenic activity of OEC in Shh- or VEGF-treated cultures was comparable. In the context of currently discussed prevascularisation strategies, which aim to include preformed vascular structures and result in perfused vascular structures after implantation (Fuchs *et al*., 2009a), Shh might be used to accelerate and to improve the formation of angiogenic structures. In addition, Shh could be beneficial in reducing the effective pre-culture time of prevascularised tissues *in vitro*. Our previous studies demonstrated that the formation of microvessel-like structures of blood-derived outgrowth endothelial cells in co-culture is usually initiated within 1 week of co-culture and proceeds with ongoing culture time in cultures in EGM-2 with no additional growth factors (Fuchs *et al*., 2007; Fuchs *et al*., 2009b).

Besides this potential to induce vascular structures already after a short time of incubation, Shh also offers the advantage of stimulating not only the release of VEGF. Several studies including this present one have shown that Shh also stimulates angiopoietin-1 and -2 (Pola *et al*., 2001; Dohle *et al*., 2010). This in turn might be responsible for the promotion of the angiogenic activation of OEC in the co-culture compared to VEGF stimulation alone. We have previously shown that Shh improves VEGF and angiopoietin-1 expression and secretion by osteoblasts, as well as angiopoietin-2 derived from OEC in the co-culture system (Dohle *et al*., 2010). This observation is in accordance with reports from the literature that angiopoietin-1 is mainly expressed by mesenchymal cells and perivascular cells like pericytes or smooth muscle cells and acts in a paracrine manner on the endothelium. On the other hand angiopoietin-2 is expressed by endothelial cells themselves, suggesting an autocrine function (Stratmann *et al*., 1998; Fiedler *et al*., 2004; Scharpfenecker *et al*., 2005). In the present study protein concentrations of angiopoietin-1 and angiopoietin-2 are significantly higher in Shh treated co-cultures compared to untreated controls. Nevertheless, VEGF treatment of co-cultures leads in comparison to Shh and untreated controls to a significant increase of angiopoietin-2 in supernatants at both time points of investigation. The roles of angiopoietin-1 and angiopoietin-2 in terms of angiogenesis seem to be pleiotropic. The precise balance between both molecules, both competing for the Tie-2 receptor, is essentially required to control the angiogenic activation of endothelial cells as well as the maturation of newly formed vessels. Angiopoietin-2 competes with angiopoietin-1 and thus acts as an inhibitor of the angiopoietin-1/Tie signalling pathway (Maisonpierre *et al*., 1997). From the literature it is known that angiopoietin-1 stimulates vessel growth in different tissues by mobilising endothelial progenitor cells and by inducing the recruitment of pericytes (Suri *et al*., 1996; Visconti *et al*., 2002). In addition, the angiopoietin-2-related angiogenic control mechanisms seem to be highly complex. Angiopoietin-2 acts as a pro-angiogenic co-factor in collaboration with VEGF but also induces endothelial cell death when VEGF is absent (Visconti *et al*., 2002). By comparing the effects of Shh and VEGF treatment on the co-culture system, it appears that VEGF treatment shifts the balance of the two angiopoietins in the system in favour of angiopoietin-2. This could explain the similar angiogenic activity of OEC in co-cultures at the later time point (14 days) independent of whether the co-cultures have been treated with VEGF or Shh. On the other hand this shift in balance towards angiopoietin-2 could also lead to a failure in terms of vessel stabilisation, as described in the following sections.

In comparison to VEGF, Shh treatment of co-cultures leads to an upregulation of the smooth muscle cell-associated markers, α-smooth muscle actin, desmin and myocardin at the mRNA level at both time points of investigation. In accordance with these observations the total protein amount of α-smooth muscle actin determined in western blots was higher in response to Shh stimulation, both after 24 h and 14 days. Nevertheless, also in untreated controls we observed indications for vessel stabilisation in terms of smooth muscle cell expression, which in contrast to VEGF treatment proceeded with time of culture and which were also supported by morphological findings. In the present study cryosections showed the association of OEC-derived vascular structures with cells staining positively for smooth muscle actin in a pericyte-like localisation, as well as the presence of basal membrane components laminin and collagen type IV as indicators of vessel maturation.

These findings and the associated mechanisms were further specified by the investigation of growth factors that lead to the coverage of newly formed vessels with mural cells. In addition to tie/angiopoietin described before, at least two other different essential signalling pathways are activated during vessel stabilisation, namely the PDGFB/PDGFRβ pathway and TGF-β signalling system (Hellstrom *et al*., 2001; Gaengel *et al*., 2009). In this present study Shh treatment for 24 h leads to a significant increase of PDGF-BB in the supernatants of OEC monocultures, as well as a significant upregulation of PDGF-BB at the mRNA level after 24 h and after 14 days of Shh treatment. Platelet-derived growth factor (PDGF-BB) plays a well defined role during the recruitment of pericytes to the nascent blood vessels (Andrae *et al*., 2008). PDGF-BB is secreted from angiogenic sprouting endothelial cells, where it serves as a chemoattractant for PDGF-BB receptor (PDGFRβ) expressing pericytes or

smooth muscle cells (Zerwes and Risau, 1987; Shinbrot *et al*., 1994; Lindahl *et al*., 1997). Additionally, PDGF-BB also induces the differentiation of undifferentiated mesenchymal cells towards a mural cell fate (Hellstrom *et al*., 1999; Hirschi *et al*., 1999).

In addition, transforming growth factor beta (TGF-β)-mediated signalling pathways play an important role in the interaction between mural cells/pericytes and endothelial cells (Dickson *et al*., 1995). TGF-β acts in a pleiotropic manner and dose-dependently on the angiogenic process. At low concentrations TGF-β can contribute to angiogenic activation by the upregulation of pro-angiogenic molecules and proteases. At a high dose TGF-β can also inhibit endothelial cell proliferation and promote the recruitment or differentiation of smooth muscle cells at sites of *de novo* blood vessel formation (Goumans *et al*., 2002; van den Driesche *et al*., 2003). In response to Shh TGF-β was also upregulated significantly after 24 hours of treatment compared to the treatment with the commonly used VEGF and untreated controls.

Blocking experiments using the Shh pathway inhibitor cyclopamine underlined the correlation of Shh and factors involved in vessel stabilisation in our *in vitro* system. In the presence of both, Shh and cyplopamine, Ang-1, TGF-β and PDGF-BB gene expression levels were tentatively reduced in a concentration dependent manner. In addition, effects of Shh on the smooth muscle actin expression were also influenced by the inhibitor cyclopamine. If Shh in comparison VEGF has the ability to improve both, the angiogenic activity and vessel maturation *in vivo*, has to be addressed in future experiments. Nevertheless, the present study supports the assessment of relevant factors in response to Shh in a more simplified experimental setting but also takes into account the cellular crosstalk by the co-culture approach.

In conclusion, Shh compared to VEGF treatment results in a beneficial effect on the angiogenic activity of endothelial cells, resulting in microvessel-like structures even after short time exposure periods, which might be helpful in accelerating the vascularisation process. In addition, in contrast to VEGF Shh also favours several factors involved in vessel stabilisation *in vitro*, which is one of the key factors in establishing a long-lasting and stable vascularisation. The current findings are based on an *in vitro* model which has the potential to permit further insight into underlying mechanisms of bone vascularisation and could also support the identification of factors stimulating the vascularisation process in therapeutic approaches. Further evidence is presented that sonic hedgehog could be one of the potential beneficial factors.

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Editor's Note: All questions from the reviewers were answered by text changes. Hence, there is no "Discussion with Reviewers" section.