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ORIGINAL ARTICLE

Differential osteopontin expression in human osteoblasts derived from iliac crest and alveolar bone and its role in early stages of angiogenesis

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Abstract

In our previous study, we revealed significant differences of osteopontin (OPN) gene expression in primary human osteoblasts (HOBs) derived from iliac crest bone (iHOBs) and alveolar bone (aHOBs). The present study aims at assigning this discriminative expression to a possible biologic function. OPN is known to be involved in several pathologic and physiologic processes, among others angiogenesis. Therefore, we studied the reaction of human umbilical vein endothelial cells (HUVECs) to HOB-derived OPN regarding angiogenesis. To this end, human primary explant cultures of both bone entities from ten donors were established. Subsequent transcription analysis detected higher gene expression of OPN in iHOBs compared to aHOBs, thereby confirming the results of our previous study. This difference was particularly apparent when cultures were derived from female donors. Hence, OPN protein expression as well as the angiogenic potential of OPN was analyzed, originating from HOBs of one female donor. In accordance to the gene expression level, secreted OPN was more abundant in the supernatant of iHOBs than in aHOBs. Moreover, secreted OPN was found to stimulate migration of HUVECs, but not proliferation or tube formation. These results indicate an involvement in very early stages of angiogenesis and a functional distinction of OPN from HOBs derived from different bone entities.

Keywords Alveolar osteoblast · Iliac crest osteoblast · Osteopontin · Angiogenesis · Human umbilical vein endothelial cells

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Introduction

Osteopontin (OPN) is an acidic phosphorylated glycoprotein, which belongs to the protein family of "Small Integrin-Binding Ligand N-linked Glycoproteins" (SIBLINGs) and plays a decisive role in the biology of mineralized tissues such as bone and teeth [1]. Although first isolated from bovine bone matrix in 1985, OPN is widely expressed in various mammalian tissues such as brain, lung, placenta, gastrointestinal tract, kidney and angiogenic tissues such as endothelium [2–4]. There is growing evidence that OPN exerts multiple functions and is involved in different cellular processes, including angiogenesis, mineralization, hematopoiesis, inflammation, autoimmune diseases, obesity, tumorigenesis and metastasis [2, 5–7]. In addition to the mineralization process, hematopoiesis and angiogenesis are in direct relation to the bone physiology regulated by OPN [1]. In the hematopoietic context, angiogenesis, which implies the formation of new blood vessels from preexisting vessels, is critical for the development and healing of bone

tissue. This is imperative not just for the nutrition and oxygen supply of osteogenic cells, but also for the recruitment of precursor cells to sites of osteogenesis [8–10]. Under in vitro conditions, endothelial-directed angiogenesis is divided into migration, proliferation and differentiation [9, 11]. In the angiogenic context, OPN is known to play a decisive role in regulating proliferation, motility, migration and tube formation of human umbilical vein endothelial cells (HUVECs) through its binding to integrin receptors [5, 12]. Numerous studies revealed that the multifunctionality of OPN relies on alternative splicing and different posttranslational modifications, e.g., phosphorylation, glycosylation, proteolytic cleavage and polymerization. In addition, OPN could be located intra- or extracellularly (membrane associated or secreted) [2, 13–15]. Three OPN splice variants are known: OPNa (full-length), OPNb (lacking exon 5) and OPNc (lacking exon 4). These splice variants are known to have different physiologic functions regarding tumor progression as well as different physical properties, e.g., solubility [15, 16]. In a recent study, we examined the different gene expression profiles of bone-related genes in primary human osteoblasts (HOBs) of alveolar bone (aHOBs) and of iliac crest (iHOBs) obtained from three female donors. Herein, we identified the distinct discriminative relative gene expression of OPN between iHOBs and aHOBs as a striking molecular characteristic, in agreement with the findings described by Lee and colleagues [17, 18]. Although iHOBs and aHOBs are both descendants of mesenchymal progenitors, and are destined to bone formation, they differ in their developmental origin and their ossification processes [19]. Many cranial bones, e.g., alveolar bone, derive from neural crest cells and ossificate intramembranously [20]. During this process, mesenchymal progenitors condense and differentiate directly into osteoblasts [19, 20]. Most of the appendicular skeleton, e.g., iliac crest, originates from the mesoderm and ossificates endochondrally, i.e., the mineralization process takes place via an intermediate cartilaginous stage [21, 22]. Skeletal site-specific variations have been described in comparative in vitro studies with bone lineage cells obtained from endochondral and intramembranous bone. For instance, the proliferation, mineralization capacity and expression of bone gene markers differ remarkably between these two bone types [17, 18, 23–25].

It is necessary to emphasize that dexamethasone was not added to the mineralization medium of the HOBs in contrast to our previous study. OPN expression is known to be influenced by glucocorticoids, e.g., dexamethasone, which is a common substitute of mineralization media, enhancing the differentiation of HOBs [26, 27]. In the present study, we confirm the discriminative OPN gene expression between iHOBs and aHOBs for ten further donors. Next, we confirmed these striking OPN gene differences on the protein level. We revealed discriminative levels of secreted OPN protein for iHOBs and aHOBs, obtained from one female donor, who exhibited the highest differences in OPN transcription. Furthermore, we demonstrated for the first time that secreted OPN of iHOBs is directly involved in the promotion of HUVEC migration, while secreted OPN did not affect HUVEC proliferation and tube formation. These results indicate that iHOBs and aHOBs differ in their OPN secretion level and thereby exhibit different biofunctional roles in the context of the early stages of angiogenesis.

Materials and methods

Isolation and culture of primary human cells

Primary human osteoblasts (HOBs) were isolated from the alveolar bone and iliac crest of five healthy female and five healthy male donors as previously described [17]. All specimens were obtained under informed consent according to the protocols of the Committee of Ethics of the Charité, Berlin, and the University Medical Center Freiburg, Germany. The donors had no systemic disease and did not use regular medication. Due to the limited availability of cells, we focused on the downstream experiments on osteoblasts derived from one female donor, which exhibited the highest difference in OPN transcription. To ensure the osteoblastic origin of the outgrowing cells, the mineralization capacity of the cells was tested with alizarin-red staining as previously described [17]. Initially, 15,000 cells/cm^2 were seeded in 12-well plates (Greiner Bio-One, Frickenhausen, Germany) and cultivated in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Darmstadt, Germany) supplemented with 10% (w/v) fetal calf serum (FCS, Biochrom, Berlin, Germany), 2 mM l-alanyl-L-glutamine (Life Technologies) and 0.1 mg/ml kanamycin (Sigma-Aldrich, Taufkirchen, Germany). Extracellular matrix mineralization was induced by incubating confluent cultures with mineralization medium (MM) consisting of GM supplemented with 50 µg/ml ascorbic acid (Sigma-Aldrich) and 10 mM β-glycerol phosphate (Sigma-Aldrich). All experiments were carried out with confluent HOB cultures incubated for 7 days with MM. To produce OPN-containing HOB supernatants for protein analysis and experiments concerning the angiogenic effect of OPN, MM was exchanged with serum-free MM at day 7 and cells further cultured for 24 h under serum-free conditions. Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell (Heidelberg, Germany) and cultivated at 37 °C with 5% CO_2. The culture medium consisted of endothelial cell growth medium (EM, PromoCell) supplemented with 5% (w/v) FCS (Biochrom) and 0.1 mg/ml kanamycin (Sigma-Aldrich). All experiments were carried

out with HOBs of passage 5 and HUVECs between passage 4 and 6.

OPN gene expression analysis

For gene expression analysis of OPN in ten donors of different genders, confluent HOBs were cultured according to our previous study [17] for 7 days in MM (see also the section above) and then lysed for total RNA extraction. To analyze the influence of culture time on gene transcription, we subsequently performed further experiments with HOBs of one donor that showed the highest differential OPN expression between aHOBs and iHOBs. The cells were cultured as described above and RNA extracted at day 7, 14, 21 and 28. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and analyzed for concentration and integrity by capillary electrophoresis (Experion Automated Electrophoresis System, Bio-Rad Laboratories, Munich, Germany). RNA was reverse transcribed into cDNA using the RevertAid RT Kit (Life Technologies). Real-time qPCR reactions were carried out with the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories) using RT^2 SYBR Green qPCR Master Mix (Qiagen) and cDNA equivalent to 10 ng of total mRNA. In addition to the commercially available OPN RT^2 qPCR Primer Assay (Genbank: NM_000582, Qiagen), designed primers were used to determine the relative expression of OPN splice variants: OPNa (Genbank: NM_001040058.1, forward: ATCTCCTAGCCC CACAGAAT, reverse: CATCAGACTGGTGAGAATCAT), OPNb (Genbank: NM_000582.2, forward: ATCTCCTAG CCCCACAGA, reverse: AAAATCAGTGACCAGTTC ATCAG), OPNc (Genbank: NM_001040060.1, forward: TGAGGAAAAGCAGAATGCTG, reverse: GTCAAT GGAGTCCTGGCTGT) [13]. By using the annealing temperature gradient and melting curve analysis, the annealing temperature was determined for each splice variant primer pair: OPNa (60 °C), OPNb (62 °C) and OPNc (60 °C). The housekeeping genes beta-actin (ACTB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls in all examined cell types. Differences in gene expression level were analyzed with the ΔΔCt method by normalizing iHOB gene expression to the respective gene expression in aHOBs. To support the qPCR results of the splice variants, a conventional PCR with 10 ng cDNA was conducted in 30 cycles with Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions.

OPN Western Blot

To examine the OPN expression in HOBs on the protein level, we performed Western blot analysis of cell lysates and supernatants derived from osteoblast cultures. Therefore, HOBs were cultivated for 7 days in MM and then for a further 24 h in serum-free MM. After that, HOBs were rinsed with cold phosphate-buffered saline (PBS, Gibco, Life Technologies), lysed for 30 min on ice in 250 µl RIPA buffer (Sigma-Aldrich) and centrifuged at 18,000*g* for 10 min. The supernatants of the respective HOB cultures were collected and likewise centrifuged at 18,000*g* for 10 min. The protein concentration of the cell lysates and the supernatants was measured using the Pierce BCA Protein Assay Kit according to the manufacturer's protocol (Life Technologies). For Western blot analysis, 10 µg of total protein of the cell lysate and 3 µg of total protein of the supernatant, respectively, were separated by SDS-PAGE on 4–15% Criterion TGX Stain-Free precast gels (Bio-Rad Laboratories). Proteins were transferred to PVDF membranes (Bio-Rad Laboratories) using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). After blocking with Tris-buffered saline (TBS, Bio-Rad Laboratories) containing 5% (w/v) bovine serum albumin (BSA, Sigma Aldrich) and 0.2% (w/v) Tween (Sigma-Aldrich) for 1 h at room temperature, membranes were incubated overnight at 4 °C with monoclonal mouse anti-OPN antibody (1:5000, Sigma-Aldrich) or mouse anti-GAPDH antibody (1:5000, Abcam, Cambridge, UK) in TBS containing 0.5% (w/v) BSA and 0.2% Tween. After incubation for 1 h at room temperature with HRP-labeled secondary antibody (1:3000, Li-Cor Biosciences, Homburg, Germany), proteins were detected using the Western-Sure ECL substrate (Li-Cor Biosciences) and the Chemi-Doc Touch imager (Bio-Rad Laboratories).

OPN ELISA

To determine the OPN concentration in cell lysates and supernatants of HOBs, we used a human Osteopontin ELISA Kit (Sigma-Aldrich), which contained a mouse monoclonal OPN antibody that detects the amino acids 17–300 of OPN. The cell lysates and supernatants of HOBs were collected and prepared as already described for the Western blot analysis. The measurement of the total protein concentration in the samples was done with the Pierce BCA Protein Assay and normalized to the 10-µg total protein amount used for ELISA analysis. Furthermore, we determined the neutralization efficiency of a polyclonal goat anti-OPN-neutralizing antibody (R&D Systems, Wiesbaden-Nordenstadt, Germany), which was used to examine the effect of OPN on HUVEC migration,

proliferation and tube formation. For this, 5 µg/ml of the neutralizing antibody was added to the HOB supernatants, incubated for 30 min and analyzed by OPN-ELISA.

HUVECs cell migration assay

HUVEC migration was analyzed using Cell Invasion/Migration Plate (CIM plates, OMNI Life Science, Bremen, Germany) and xCelligence Real-Time Cell Analyzer (OMNI Life Science). This approach enables the real-time monitoring of cell migration by impedance spectroscopy. Prior to cell seeding, the microporous membranes of the CIM plate wells were coated with 40 µl fibronectin per well (10 µg/ml, PromoCell) and incubated at 37 °C for at least 3 h. For migration analysis, 20,000 HUVECs in serum-free MM were seeded in the top chamber of the CIM plates and (1) supernatants from HOBs with or without OPN-neutralizing antibody (5 µg/ml), (2) EM as positive control or (3) serum-free MM as negative control were added to the lower chamber. The HOB supernatants used for the migration assay were treated as described for the Western blot analysis (centrifugation at 18,000*g* for 10 min). After 30 min equilibration at 37 °C, the cell index was continuously measured for 20 h. For the analysis of the cell migration, data obtained at 20 h were used [28].

Indirect immunofluorescence image analysis for Ki-67

To determine whether HOB-derived OPN has an impact on HUVEC proliferation, we performed immunofluorescence staining of the proliferation marker protein Ki-67 in HUVECs after 24 h incubation with (1) supernatants from HOBs with or without OPN-neutralizing antibody (5 µg/ml), (2) EM as positive control or (3) serum-free MM as negative control. For this purpose, 10,000 cells per well were seeded in collagen 1-coated 8-Well Immunofluorescence Chambers (Ibidi, Martinsried, Germany) and incubated for 24 h in EM. The medium was then replaced by the aforementioned test media, namely HOB supernatants, EM or serum-free MM, and cultured for a further 24 h. For Ki67 immunofluorescence staining, cells were fixed with 99.8% ethanol (Honeywell Speciality Chemicals Seelze, Seelze, Germany) for 10 min on ice and treated with 5% (w/v) BSA (Sigma Aldrich) and 0.1% (w/v) Triton X-100 (Sigma Aldrich) in PBS for 30 min at room temperature. The primary rabbit anti-Ki-76 antibody (1:200, Abcam) and secondary fluorochrome-conjugated antibody Alexa Fluor 594 goat anti-rabbit IgG (1:200, Life Technologies) were diluted in PBS containing 0.5% BSA and the samples incubated with the antibodies for 1 h at room temperature. Nuclei were stained with 300 nM 4′,6-diamidin-2-phenylindol (DAPI, Life Technologies) for 10 min at room temperature. The samples were embedded in Fluoromount mounting medium (SouthernBiotech, Birmingham, USA) and analyzed with the Biozero BZ-8000 fluorescence microscope (KEYENCE, Neu Isenburg, Germany).

To evaluate the proliferation rate in HUVECs, the ratio between the Ki-67-expressing cells and total amount of cells visualized by DAPI staining was calculated (at least 200 cells per treatment group).

HUVEC tube formation assay

The influence of HOB supernatants on the formation of tube-like structures by HUVECs cultured on a basement membrane-like matrix was examined on µ-Slide Angiogenesis coverslips (Ibidi) coated with 10 µl growth factor reduced Matrigel (10 mg/ml; Becton-Dickinson, Heidelberg, Germany).

HUVECs were resuspended in the (1) supernatants from HOBs with or without OPN-neutralizing antibody (5 µg/ml), (2) EM (positive control) or (3) serum-free MM (negative control) and 10,000 cells per well seeded on Matrigel-coated µ-Slides. After 24 h incubation, phase contrast images were taken using an inverted Leica DM IL microscope. Quantitative analysis of the formed tube network was performed by measuring the number of nodes connecting at least three neighboring cells and the length of tubes using the Angiogenesis Analyzer Plug-In for Image J (Gilles Carpentier, Faculté des Sciences et Technologie, Université Paris Est, Creteil Val de Marne, France).

Statistical analysis

All experiments were performed in three biologic replicates with at least three technical replicates except the gene expression analysis of the ten donors. Data are expressed as mean values ± standard deviation (SD) and were compared for statistically significant differences using the unpaired Student *t* test ($p < 0.05$) (GraphPad statistical software, La Jolla, CA, USA).

Results

Elevated levels of OPN transcripts discriminate iHOBs from aHOBs and are attributed to secreted OPN on the protein level

As recently published, qPCR array analysis revealed striking differences in OPN gene transcription in iHOBs with matched aHOBs derived from three female donors [17]. To confirm these differences in relative OPN gene expression, we analyzed the OPN transcription levels of iHOBs and aHOBs, obtained from a further five female and five male

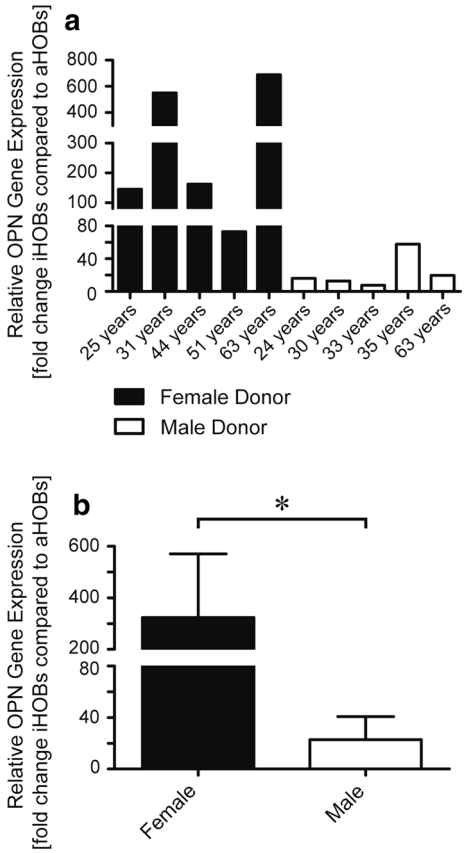

Fig. 1 Relative OPN transcriptional analysis discriminates iHOBs and aHOBs, thereby revealing relative gender-depending OPN expression differences. **a** Generally increased OPN expression in iHOBs compared to aHOBs derived from ten donors (5 females and 5 males) at day 7. **b** These relative OPN gene expression results were summarized by gender. OPN expression was significantly more pronounced in females; 10 ng RNA was used for the relative gene expression analysis and normalized to the housekeeping genes ACTB and GAPDH, while the gene expression of equivalent aHOBs was used as a control. Positive fold change values mean higher expression in iHOBs compared to aHOBs. Error bars represent SD, $*p < 0.05$

human donors (Fig. 1a) at day 7. An important difference from our previous study is the avoidance of the glucocorticoid dexamethasone as it could influence the OPN gene expression [26, 27]. Despite the lack of dexamethasone in the MM, the OPN gene expression analysis revealed that the iHOBs of all donors displayed generally elevated OPN transcription levels, ranging from 5- to 700-fold, compared to the respective aHOBs derived from the same donor (Fig. 1a). Intriguingly, relative OPN transcription was also found to differ between the genders, showing overall higher expression levels in iHOBs derived from female donors (Figure 1b). To examine the influence of culture time on OPN gene expression, we next analyzed transcription levels at 7, 14, 21 and 28 days in HOBs derived from the 31-year-old female donor, who showed the highest differences in OPN expression between both osteoblast entities and provided sufficient iHOB amounts for the downstream experiments (see also Fig. 1a). It should be pointed out here that the surgical removal of bone tissue samples from different tissue origins from the same donor is very rare and only possible during special surgical interventions such as jawbone augmentations. This in turn resulted in some experimental limitations regarding the cell availability of iHOBs. Moreover, iHOBs generally exhibit a long generation time and rapidly reach senescence [17]. This leads, depending on the donor bone, to low cell yields that limit the number of experiments per donor.

The analysis of time dependence of OPN expression revealed constitutively significant elevation of transcripts in iHOBs with matched aHOBs at all times of investigation (Fig. 2a). Intertemporal comparison showed the highest difference between both bone cell entities at day 7, as indicated by a 556-fold higher OPN expression (see Fig. 2a). Based on these results, all subsequent experiments were performed with HOBs cultured for 7 days after reaching confluency. Regarding the expression levels of the different OPN splice variants, OPNa, which encodes for full length OPN, was present at significantly higher amounts in iHOBs compared to aHOBs. Besides, OPNb and OPNc were only detectable in iHOBs as mean CT values of 35 and above for OPNb and OPNc in aHOBs were scored negative or below the detection limit (see also table in Fig. 2b). As shown in Fig. 2c, analysis of the PCR products generated after 30 cycles with the customized primers corroborated these findings. OPNa (300 bp), OPNb (250 bp) and OPNc (100 bp) were clearly detectable in iHOBs, while transcripts were missing in aHOB counterparts. The visible band at a molecular weight of 300 bp with OPNc primers in the aHOB sample probably results from unspecific binding of the primer to OPNa cDNA.

To clarify whether the observed transcriptional differences in OPN expression can also be found for the protein level, we next analyzed supernatants and cell lysates of HOBs by Western blot and ELISA. To ensure that the supernatants contained exclusively secreted proteins of HOBs, culture medium was replaced by serum-free medium at day 7 and cells cultured for further 24 h prior to harvesting. As shown in Fig. 3, Western blot analysis of the total cell lysates led to almost equal band intensities and thus to fairly comparable OPN amounts in iHOBs and aHOBs (Fig. 3a). By contrast, supernatants from iHOBs and aHOBs revealed striking differences in OPN expression, characterized by higher protein levels with emphasis on a molecular weight of 65 kDa (Fig. 3b). The difference between the protein bands, running at a molecular weight of 75 kDa, was also visible, but less pronounced. In addition, the iHOB supernatant contained proteins with lower and higher molecular weight, detected in the known OPN range, spanning from 35 kDa to 250 kDa.

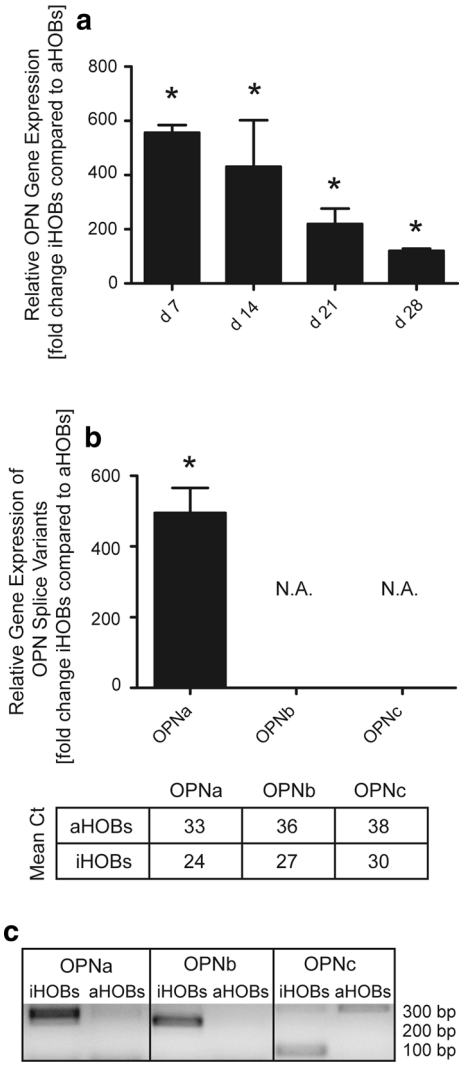

Fig. 2 Relative gene expression analysis of OPN and its splice variants of one female donor (31 years) conducted with qPCR and conventional PCR. **a** Time point analysis revealed constitutively significantly higher OPN gene expression in iHOBs with matched aHOBS at 7, 14, 21 and 28 days. **b** Relative gene expression with customized OPNa, OPNb and OPNc primers at day 7. The splice variants OPNb and OPNc were only present in iHOBs. OPNa was significantly more highly expressed in iHOBs compared to aHOBs. The table below the bar chart depicts the mean cycle threshold (Ct) values of the three OPN splice variants in aHOBs and iHOBs. **c** PCR analysis with 30 cycles was conducted to detect the splice variants OPNa (300 bp), OPNb (250 bp) and OPNc (100 bp) of HOBs. These results confirmed the qPCR analysis results and revealed only a weak band of OPNa for aHOBs; 10 ng RNA was used for the conventional PCR and qPCR analysis, and for the relative qPCR the OPN gene expression was normalized to the housekeeping genes ACTB and GAPDH, while the gene expression of equivalent aHOBs was used as a control. Positive fold change values mean higher expression in iHOBs compared to aHOBs. Error bars represent SD, $*p < 0.05$

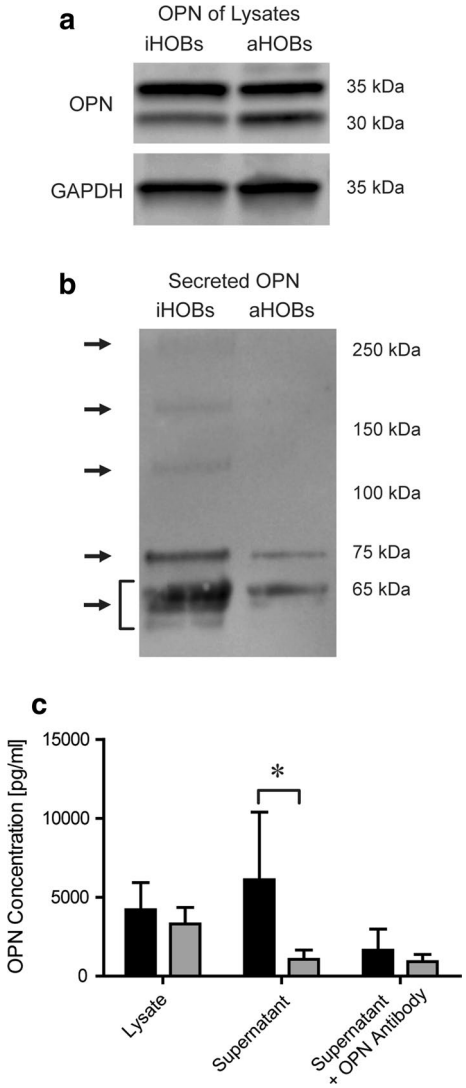

Fig. 3 OPN protein expression in the cell lysates and supernatants of HOBs of one female donor (31 years) at day 7 determined by Western blot analysis (**a**, **b**) and ELISA (**c**). The OPN protein levels in cell lysates were comparable (**a**), while the supernatant of iHOBs and aHOBs revealed differences in the OPN amount (**b**). In the supernatant of iHOBs, the bands of OPN were more abundant at 65 and 75 kDa. Additionally, the OPN splice variants (below 65 kDa) and polymerized OPN proteins (above 75 kDa) were only present in iHOBs. **b**, **c** OPN concentrations of the cell lysates and supernatants of HOBs and the neutralization efficiency of an anti-OPN antibody (5 µg/ml) were analyzed using ELISA. The results confirmed the Western blot analysis because of the comparable OPN amount in the cell lysates and the significant OPN differences in secreted OPN between iHOBs (black bars) and aHOBs (gray bars). Additionally, the efficiency of the neutralizing-OPN antibody was verified. Error bars represent SD, $*p < 0.05$

These proteins most likely represent the splice variants in case of the lower molecular weight band and the various OPN polymers in case of high-molecular-weight bands (Fig. 3b) [29–31]. This trend in OPN protein amounts observed in cell lysates and supernatants was confirmed by ELISA. In detail, OPN concentrations in cell lysates of both HOB entities were comparable and ranged from 3300 to 4200 pg/ml (Fig. 3c). By contrast, supernatants of

iHOBs contained approximately 6100 pg/ml OPN and thus an almost sixfold higher OPN protein amount compared with aHOB supernatants, which contained about 1100 pg/ml. These differences, observed for OPN in the examined HOBs, clearly identified the supernatant of iHOBs and aHOBs as their specific distinguishing factor. Furthermore, the addition of an OPN-neutralizing antibody (5 µg/ml) led to a clear decrease of the OPN-antigen signal in the supernatant, particularly in that of iHOBs (Fig. 3c). As the coated antibody of the ELISA detects nearly the entire OPN protein (17–300 aa), this neutralization-antibody-based approach was reasonable to examine the putative role of OPN on early stages of HUVEC angiogenesis in the subsequent experiments.

Biofunctional discrimination of iHOBs from aHOBS by significant OPN-promoted HUVEC migration

Since all the OPN splice variants (OPNa, OPNb and OPNc) have been shown to be involved in angiogenesis, we were motivated to analyze whether (1) HOB-derived OPN affects angiogenesis and, if so, (2) whether different OPN protein amounts, which depend on the originating cell type, have an impact on this issue [13, 16, 32, 33]. To this end, we exposed HUVECs, which represent a commonly used angiogenic in vitro model, to cell culture supernatants derived from HOBs. As previously demonstrated by Western blot and ELISA, HOB supernatants contained secreted OPN. Since endothelial angiogenesis includes endothelial cell migration, proliferation and tube formation, experiments were performed, considering each parameter separately. HUVEC migration represents a very early stage of angiogenesis and is an indispensable prerequisite. Thus, we examined the chemoattractant, i.e., migration-promoting effect of the OPN-containing HOB supernatant on HUVECs by employing CIM plates and the xCelligence Real-Time Cell Analyzer (Fig. 4). The strongest migration-promoting effect has been detected in HUVECs exposed to the positive control, namely fully supplemented EM. Hence, this strongest stimulatory effect was set to 100%, thereby serving as reference. By marked contrast, negligible migration was seen in case of the negative control, which consisted of serum-free MM. Application of HOB supernatants generally promoted HUVEC migration and yielded a significantly higher migration rate for iHOB supernatants when compared to the corresponding aHOB samples, namely 55 and 43%, respectively. The application of the OPN-neutralizing antibody led to a significant reduction in the migration rate of 18% in iHOB supernatants, whereas no inhibitory effect was detected for HUVECs exposed to aHOB supernatants. These findings demonstrate that OPN in iHOB supernatants is, along with other HOB-derived proteins, involved in the promotion of HUVEC migration.

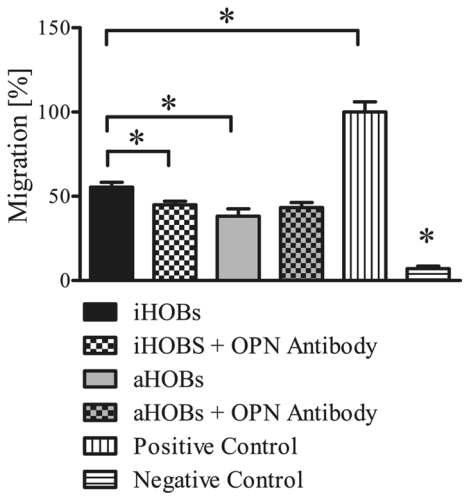

Fig. 4 To investigate the chemoattractant effect of secreted OPN in the supernatants of HOBs, HUVEC migration was analyzed using the Cell Invasion/Migration Plate and xCelligence Real-Time Cell Analyzer. For migration analysis, HUVECs were exposed to (1) the supernatant of HOBs with or without OPN-neutralizing antibody, (2) endothelial cell growth medium (positive control) or (3) serum-free mineralization medium (negative control). By using neutralizing-OPN antibody, secreted OPN of iHOBs showed a significant reduction of HUVEC migration. The cell index was continuously measured for 20 h. For the analysis, cell indices at 20 h were used. Error bars represent SD, $*p < 0.05$

OPN does not stimulate proliferation of HUVECs

Since proliferation of vascular cells also takes place in early stages of angiogenesis, we analyzed this cell behavior to examine its dependence on OPN. For this purpose, via indirect immunofluorescence staining we analyzed the expression of the proliferation marker protein Ki-67, which is related to cell proliferation due to its presence in all active phases of the cell cycle [34]. The incubation of HUVECs with the positive control (EM) led to a significantly higher amount of Ki-67-positive cells compared to the supernatant of aHOBs and the negative control (serum-free MM) (Fig. 5a, b).

In contrast to this, there was no difference in the DAPI/Ki-67 ratios of iHOB supernatants and the positive control, thus pointing to an equal efficiency in stimulating HUVEC proliferation. Furthermore, the iHOB supernatant led to a significantly higher frequency of Ki-67-positive HUVECs compared to supernatants derived from aHOBs. In both cases, the administration of the OPN-neutralizing antibody had no effect on the number of Ki-76-positive cells, indicating that OPN was not the cause for the detected differences. Thus, it appears likely that supernatant-containing

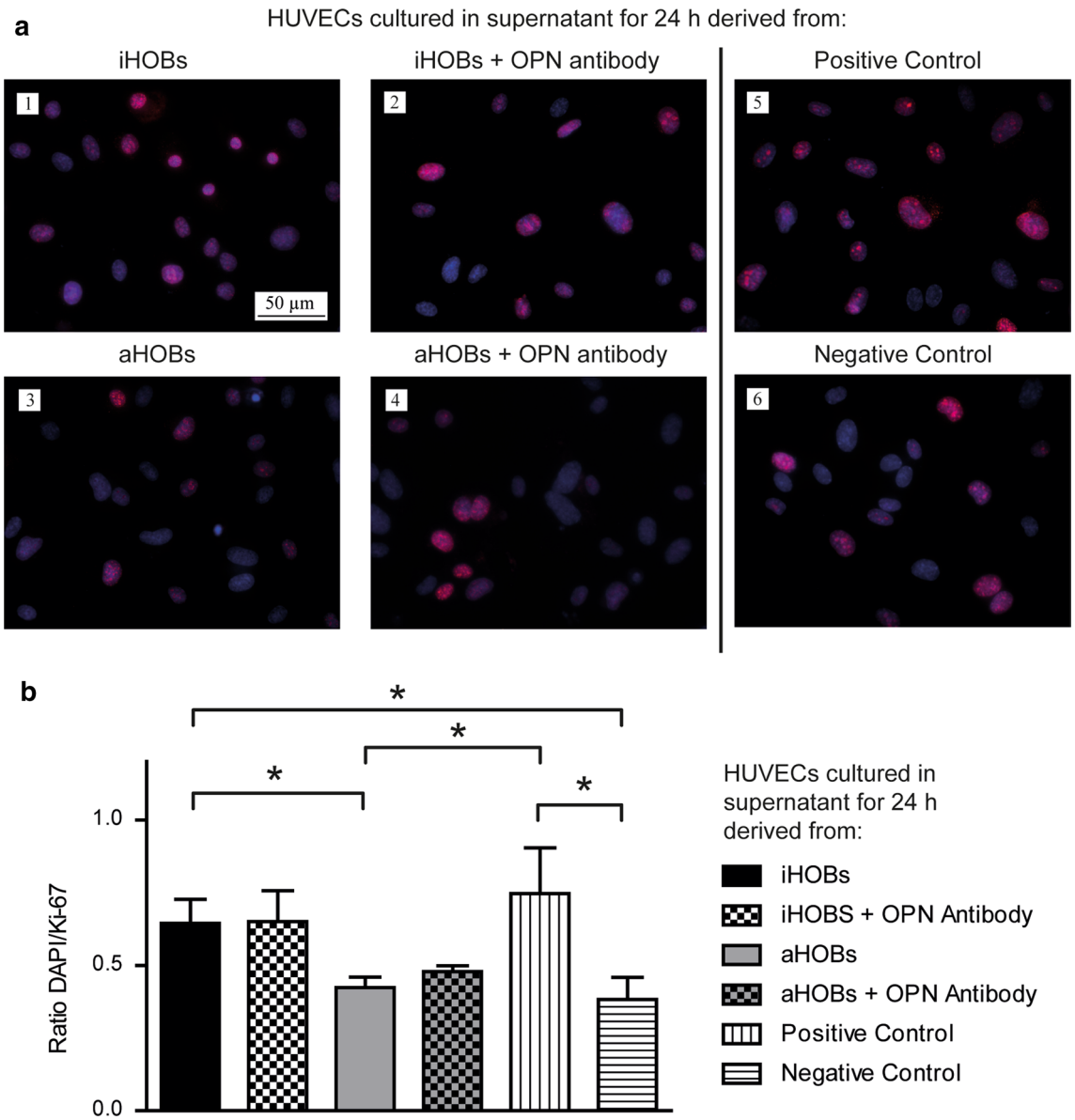

Fig. 5 Qualitative and quantitative analysis of proliferation of HUVECs exposed to (1) the supernatant of HOBs with or without the OPN-neutralizing antibody, (2) endothelial cell growth medium (positive control) or (3) serum-free mineralization medium (negative control) for 24 h. The proliferation was measured using immunofluorescence staining of the nucleus (blue) and Ki-67 (red). **a** Immunofluorescence staining revealed a higher frequency of Ki-67-positive HUVECs when exposed to the supernatant of iHOBs compared to aHOBs. The application of OPN-neutralizing antibody did not affect the frequency of Ki-67-positive cells. **b** Quantitative analysis of the immunofluorescence demonstrated significantly more Ki-67-positive HUVECs when exposed to the supernatant of iHOBs compared to aHOBs independent from the presence or inhibition of OPN. The ratio between Ki-67-expressing cells and the total number of cells was determined by cell counting of at least 200 cells per treatment group. Error bars represent SD, $*p < 0.05$

factors other than OPN are responsible for the HUVEC-targeted proliferation stimulus. Irrespective of the role of OPN in HUVEC proliferation, the observed results suggest that supernatants derived from iHOBs are generally more potent in stimulating endothelial cell proliferation than aHOB supernatants.

Secreted OPN does not influence tube formation of HUVECs

To analyze the impact of secreted OPN from HOBs on the differentiation level of HUVECs, we performed a tube formation assay. Generally, HUVECs exhibited angiogenic

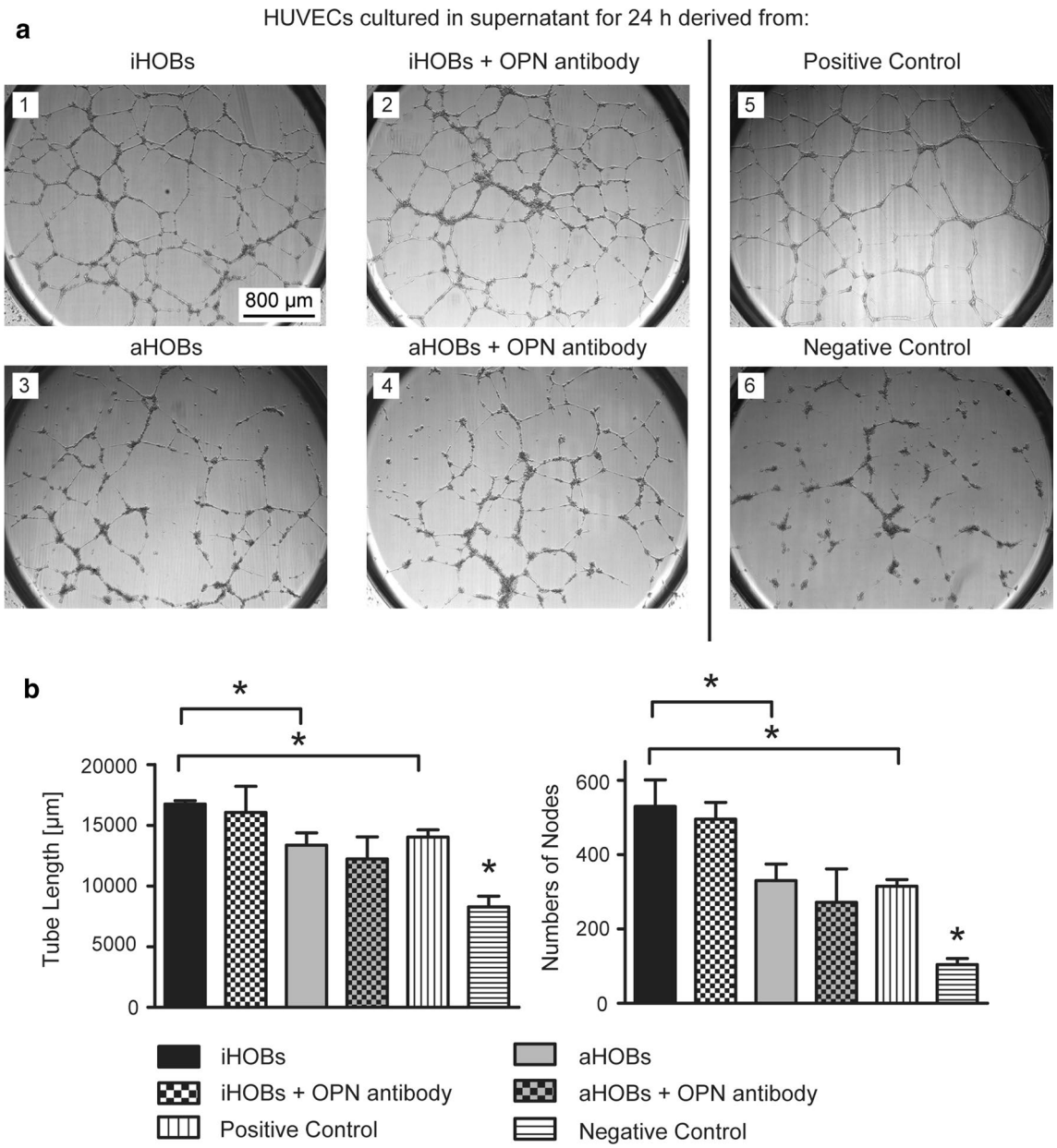

Fig. 6 Tube formation was analyzed using HUVECs resuspended in (1) supernatant of HOBs with or without OPN-neutralizing antibody, (2) endothelial cell growth medium (positive control) or (3) serum-free mineralization medium (negative control). HUVECs revealed a greater angiogenic potential when exposed to the supernatant of iHOBs compared to aHOBs. Secreted OPN had no effect on the tube formation of HUVECs. **a** The tube formation was qualitatively analyzed with phase contrast microscopy. **a** The quantification of the tube formation was measured with the "Angiogenesis Analyzer Plug-In" in Image J software. Mean values of nodes and total length of the tubes were used as read-out for analysis. The negative control was significantly lower compared to other samples. Error bars represent SD, $*p < 0.05$

potential, as evidenced by the positive and negative control via the widely accepted in vitro parameters, namely the tube length and number of formed nodes (Fig. 6b) [9]. While tubes and nodes were clearly visible in the positive control (EM), deteriorated endothelial tube structures were seen in the negative control (MM) (Fig. 6a). As already evident on the visual level, iHOB-derived supernatant induced the development of HUVECs tubes, which extended more in length compared to the aHOBs' supernatant. The same applied to the number of nodes (Fig. 6b). Interestingly, both in vitro parameters of angiogenesis remained unaffected in the presence of an OPN-neutralizing antibody, thereby leading to the assumption that tube length and node number are regulated independent of HOB-secreted OPN (Fig. 6a).

Detailed quantification of the above-described in vitro angiogenesis parameters coincided almost entirely with the qualitative visual analysis and revealed significant differences in the tube length and numbers of nodes between iHOBs and aHOBs (Fig. 6b). OPN-neutralizing antibody, however, showed no effects on either parameter, irrespective of the HOB entity. While supernatants from aHOBs exhibited a similar effect on HUVEC tube formation as the positive control, the iHOB-derived supernatants exhibited an even greater influence. This indicates that the composition of the protein mixture and/or quantity of particular proteins secreted by iHOBs makes them an effective promotor of HUVEC differentiation.

Discussion

In our recently published study, a comparative relative qPCR analysis revealed a 140-fold higher amount of OPN mRNA in iHOBs compared to aHOBs obtained from three female donors [17]. In the present study, we aimed to extend the found OPN gene expression differences between iHOBs and aHOBs to a further ten donors and also to the protein level. In addition, we analyzed the putative biofunctional role in the angiogenesis of the examined HOBs derived from one female donor. In the present and in contrast to our previous study, the experiments were performed without supplementing dexamethasone to the MM as a potential effect of this glucocorticoid on OPN gene expression may influence the results [27].

Our experiments revealed higher OPN gene expression in iHOBs compared to aHOBs for a further ten donors, also in the absence of dexamethasone in the MM. Moreover, we showed remarkable OPN transcription differences on the gene as well as protein level in iHOBs and aHOBs for one female donor. On the biofunctional level, OPN secreted from iHOBs was shown to promote HUVEC migration, thereby suggesting a role of iHOB-derived OPN in the early stages of blood vessel development.

With respect to the OPN gene expression, we detected a constitutively higher transcription in iHOBs compared to aHOBs. Regarding the five female donors, the OPN gene expression in iHOBs was up to 500-fold higher compared to the expression in their aHOB counterparts, whereas in case of the five male donors this difference in OPN gene expression was less pronounced. On the one hand, these findings indicate that differences in OPN transcription depend on the origin of HOBs. On the other hand, our results suggest a gender-dependent OPN expression level. This detected gender-dependent transcriptional discrepancy between iHOBs and aHOBs may be associated with the involvement of OPN in physiologic and pathophysiologic conditions. Such an association appears likely, as gender-depending OPN differences have been examined in several other studies. For example, high OPN levels in the serum have been associated with menopausal osteoporosis in females [7, 35].

Furthermore, our current OPN transcription analysis, which now includes ten donors of both sexes, clearly confirmed our previous results of OPN gene expression differences in iHOBs and aHOBs [17]. It seems likely that the higher OPN gene transcription level of iHOBs in comparison to aHOBs represents a general phenomenon in human cells. Although the number of ten donors seems to be low at first glance, this is one of the few studies comparing iHOBs and aHOBs derived from the same donor, thereby rendering the aforementioned general view veritable.

The OPN gene encodes for at least three splice variants, specified as OPN a, b and c, yielding proteins, which are further subjected to various posttranslational modifications. These modifications finally lead to an OPN molecular weight ranging from 5 kDa to 250 kDa [33, 36, 37]. Moreover, OPN is described as a substrate of transglutaminase II, an enzyme known to promote the OPN propensity for polymerization [38]. Western blot of OPN-containing supernatant in the iHOB and aHOB analysis revealed protein bands with different molecular weights, which can be attributed to the different forms of OPN. It is noteworthy that OPN has mainly been described to be involved in extracellular matrix mineralization [2]. Therefore, we not only conducted Western blot and ELISA analyses with the cell culture supernatant, but also with the cell lysates, which include both cytoplasmic OPN and cell membrane-bound OPN in conjunction with the matrix-bound proportion. The examination of both components revealed that the iHOBs and aHOBs showed fairly equal amounts of OPN protein in their cell lysates, while they clearly differed in their OPN level detected in the supernatant. This discrepancy strongly suggests that the observed transcriptional difference of OPN in iHOBs compared to aHOBs is mainly reflected in the secreted, i.e., humoral OPN protein level. This assumption is corroborated by the clear diminishment of the high OPN level in the iHOB supernatant in response to the administration of an OPN-specific antibody. A further proof of our assumption is evidenced by considering our previous study, revealing a similar capacity of matrix mineralization in iHOBs and aHOBs, which is known to be facilitated by cell-bound OPN, detectable only in the lysate.

OPN protein bands of various sizes were detected in the supernatant derived from iHOBs and aHOBs, which can be attributed to the above-described OPN modifications. The performed gene expression analysis of the OPN splice variants revealed that aHOBs were devoid of splice variants b and c. Therefore, the detected protein bands of 75 and 65 kDa in the aHOB supernatant are most likely ascribed to the OPNa splice variant, depicting various posttranslational modifications. The two corresponding OPN bands in

the iHOB supernatant revealed a much higher OPN protein expression, coinciding with the higher transcription rates of the OPNa splice variant in iHOBs compared to aHOBs. The additional protein bands above 75 kDa in iHOBs' supernatant may represent OPN polymers and the band below 65 kDa may encode for protein products of splice variants b and c. Although demonstrated only for one donor, the previously found discriminative levels of OPN in iHOBs and aHOBs, i.e., bone cells of different body sites, may not only apply on the gene transcription level, but also on the protein expression level.

With respect to OPN, pleiotropic effects have been described, among which cancer-related neovascularization is especially noteworthy [39]. In addition, a recent study also revealed a non-tumor-associated vascular-supporting effect of this glycoprotein, which is based on promotion of a vascular network, formed by circulating angiogenic cells in a rabbit wound-healing model [40]. Angiogenesis of blood vessels is a cellular process, which consists of initial activation, proliferation, cell migration, and finally differentiation and tissue infiltration of endothelial cells [5]. In general, angiogenesis plays essential roles during the development of mineralized tissues and fracture healing in particular for bones [8]. Based on these OPN functions, the discriminative levels of OPN, found in the supernatant of iHOBs and aHOBs, were tested for the first time regarding their putative role in the aforementioned initial and progressive stages of angiogenesis. Among the parameters that govern angiogenesis, chemotaxis plays a key role, implying attraction of cells via active locomotory processes, i.e., migration [41]. In the present study, OPN in the supernatant of iHOBs could be clearly assigned to being a biofunctional causative agent in the migration of HUVECs because of the significant reduction of migration promotion in the presence of the OPN-neutralizing antibody. Since up to now OPN-driven chemoattraction of HUVECs has only been demonstrated for recombinant OPN, we could prove for the first time such a migration-promoting effect for the native OPN molecule [5, 12, 42, 43]. In this context, it should be noted that different organisms are used as expression systems for recombinant OPN and therefore the products may not be the same as for human OPN regarding specific splice variants and/or posttranslational modifications, i.e., glycosylation, which might be mandatory for human-specific OPN functions in human cells [5]. Another interesting fact in the context of cell migration arises from the discriminatively expressed OPN splice variants, with lacking variants b and c in aHOBs. These splice variants are devoid of exons 4 and 5, respectively, and this lack was shown to improve OPN solubility. This greater OPN solubility of splice variant b and c, which are only present in iHOBs, may explain the higher OPN protein amount detected in iHOB supernatant. Splice variants and polymeric versions of OPN, which are exclusively found in the iHOB supernatant, have been demonstrated to have a higher affinity to migration-related integrin receptors. These receptors include integrin alpha v beta 3 in case of splice variant c and integrin alpha 3 beta 1 as well as integrin alpha 9 beta 1 in case of OPN polymers [30, 32, 36, 37, 43]. Against this background, the difference in OPN splice variants and OPN polymers expression between iHOB-compared to aHOB-derived supernatants might be causative for the significant increase of HUVEC migration. The subjection of HUVECs to the bone cell supernatants led to a clear discrimination of iHOB and aHOB supernatant regarding their ability to promote tube formation, whereby the iHOB supernatant proved to be superior. Interestingly, this superiority could not be assigned to OPN, as this effect was not altered by the administration of the OPN-neutralizing antibody. This finding is in contrast to the tube-supportive effect of OPN, demonstrated in a study by Dai et al. [5]. However, these opposing results can be explained by the different sources of OPN, which in our study implies the native molecule, while recombinant OPN was used in the aforementioned study. The fact that OPN inhibition did not affect the HUVEC-based tube formation clearly points to the presence of other molecules in the supernatant of iHOBs, which are responsible for the observed promotion of tube formation.

Another cell behavioral response, which was found to occur in an OPN-independent manner, was HUVEC proliferation, belonging to the early phases of angiogenesis in conjunction with migration. This OPN-independence was revealed by a consistent frequency of Ki-67-labeled cells and thus proliferation rate of HUVECs, observed in the absence as well as presence of the OPN-neutralizing antibody, irrespective of the supernatants' source. This implies that other factors than OPN within the supernatant are responsible for the observed effect on the proliferation rate of HUVECs. Moreover, the fact that the proliferation rate significantly differed depending on the HOB origin revealed a cell behavioral response, adding to the number of detected discriminators between the two HOB entities.

The differences between iHOBs and aHOBs obtained from the same donor and seen for OPN on the gene, protein and biofunctional level suggest a common basis, which may allow the discrimination between these two bone cell entities and may be based on differences in both the developmental biology and mode of ossification. Cranial bone, including the alveolar bone, originates from the neural crest and exclusively shows intramembranous ossification. With respect to the developmental origin and mode of ossification, cranial bone, including the alveolar bone, originates from the neural crest and exclusively shows intramembranous ossification. Most of the other skeletal bones, including the iliac crest bone, derive from the mesoderm and exhibit endochondral ossification [19, 44].

Our data, obtained from the transcriptional analysis, and confirmed for one female donor on the protein level, suggest that the glycoprotein OPN acts as a molecular discriminator between iHOBs and aHOBs. By detailed analysis of the effects that emerged from the respective HOBs-derived supernatants, we succeeded in assigning biofunctions in the angiogenic context to native OPN with emphasis on the promotion of HUVEC migration. Interestingly, angiogenesis not only served to assign discrimination of HOB-derived OPN to the above-specified biofunctions, but also led to a more general distinction between the examined HOBs, as further factors within the supernatants additionally evoked different results in their ability to direct HUVEC proliferation and tube formation. In the context of our elaborated new findings, it is reasonable to consider the notion that the observed differences in iHOBs and aHOBs obtained from one donor are based on their differences in developmental biology and the mode of ossification.

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Author contributions MW conducted the experiments, collected the data, and performed the data analysis and writing. He is further responsible for the integrity of the data. DHH contributed to the conduction of the WB experiments, analysis and writing. GF supplied the HUVEC cells for the study. KN, TF and SN provided the bone debris of the alveolar bone and iliac crest and contributed constructive scientific ideas. BA and TS generated the hypothesis, supervised the experimental part and contributed with intellectual input and writing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights statement This article does not contain any studies with human participants or animals performed by any of the authors.

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