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In vitro spheroid model of placental vasculogenesis: does it work?

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Placental vascular development begins very early in pregnancy and is characterized by construction of a primitive vascular network in a low-oxygen environment. *In vitro* three-component assays of this process are scarce. In this study, a complex three-dimensional spheroid model for *in vitro* studies of placental vasculogenesis with regard to cell–cell interactions between cytotrophoblasts (CTs), villous stromal cells and endothelial precursor cells was established. Microscopic and immunohistochemical analyses of the spheroids showed structural and differentiation patterns resembling the structure and differentiation of early placental chorionic villous tissue (in regard to the expression of multiple markers cytokeratin-7, vimentin, CD34, CD31). The authenticity of this model to *in vivo* events allowed investigation of placental vascular development and trophoblast invasion under physiological and pathological conditions. Particularly enhanced spheroidal expression of SDF-1α and its receptor CXCR4, the major chemokine system in embryonic vasculogenesis, in a low-oxygen environment was detected. In addition, our model confirmed previously described invasive phenotype of trophoblasts through collagen under low- (physiologic), but not high- (pathologic) oxygen concentrations. Therefore, the three-dimensional spheroid model consisting of major placental cell types proved to be an appropriate system to investigate early placental vessel development under both physiological and pathological conditions.

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The establishment of a vascular network is one of the earliest events in embryonic/placental development and essential for fetal growth.[1] Aberrant vascular development of the placental vascular tree has been linked in a number of serious pregnancy-related complications including intrauterine growth restriction, preeclampsia or early pregnancy loss.[2–4] As epidemiological studies have revealed a strong correlation between intrauterine growth and retardation and disease mechanisms in later life, placental vascular development is an important determinant of health in adulthood.[5]

Vascularization of the human placenta occurs independently of embryonic vascular development and begins in tertiary chorionic villi composed of an outer trophoblastic layer (syncytiotrophoblasts) and an inner mesenchymal villous core.[6–8] Syncytiotrophoblasts arise from the fusion of villous cytotrophoblasts (CTs) and have important absorptive and secretory function in the developing placenta. A subset of CTs aggregate and form columns in anchoring villi. The CTs attach to the superficial portion of the uterus and subsequently invade the walls of the uterine spiral arterioles. Thus, CT invasion anchors the fetus to the mother and participates in the remodeling of maternal uterine vessels.[9–11] Inner structures of chorionic villi are infiltrated by stromal cells thereby forming an early site for blood vessel development. Interactions of endothelial/hematopoietic progenitor cells with adjacent trophoblast and villous stromal cells (VSCs) are essential for this process as well as development and maintenance of an extensive placental vasculature. As trophoblast is a rich source of angiogenic growth factors affecting hematopoietic progenitor cells, placental vascular development might be influenced by trophoblast.[8] Furthermore, angiogenic factors of trophoblastic origin seem to have an impact on cell migration, an important process of vascular development. Ang-2, eg, stimulates the migration of

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endothelial precursor cells (EPCs).[12] As early as 21 days p.c., structures within the chorionic villi termed hemangioblastic cords could be detected.[13] These structures consist of CD34-positive cells called angioblasts. Angioblasts start to cluster and reorganize to form capillary-like structures.[14] The induction of angioblasts and the assembly of primordial vessels is tightly regulated by a number of cytokines, growth factors like VEGF and FGF, components of the extracellular matrix and its receptors.[15] The angiopoietins Ang-1 and Ang-2 and their respective receptor Tie2 are responsible for a balance between stabilization and remodeling of the primary capillary plexus and for survival of endothelial cells.[16–18] These factors have been detected in early chorionic villi, as well.[19] Recently, placenta has also been shown to be a rich source of the cytokine SDF-1α/CXCL12 and its receptor CXCR4.[20,21] As regulatory processes of early placental vascular development occur in an low-oxygen environment, we recently investigated the function of the placental-derived SDF-1α/CXCR4 system under physiologically relevant oxygen tensions.[22,23] We have demonstrated that SDF-1α recruits human stem/progenitor cells to sites of placental vascular development.

Owing to the unique structure of the human placenta, available functional assays of vascularization are sparse, whereas the precise mechanisms of early placental vascularization remain unclear.[24] As endothelial progenitor cells of placental origin may contribute to different vascular pathologies in postnatal life (eg infantile hemangioma), there is a strong need to establish adequate models of placental vasculogenesis.[25]

In this study, a complex three-dimensional, three-component coculture model of developing vessels in the human placenta was successfully established and utilized for *in vitro* placental vasculogenesis- and trophoblast invasion studies in physiological relevant oxygen environment.

MATERIALS AND METHODS

Cell Isolation and Cultivation

First trimester cytotrophoblast and villous stromal cell cultures

Magnetic-activated cell sorting (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany) was applied isolate CTs from early placental tissue collected from elective abortions after written consent from the patients and approval by the Ethics Committees (JLU, Giessen, Germany, and EMAU, Greifswald, Germany) was obtained. Enzymatic digestion of minced chorionic villi fragments was conducted as described previously.[26] Cells were subsequently separated using an antibody against CD326 (Miltenyi Biotech), an epithelial cell adhesion molecule expressed by CTs. Up to 90% of the $CD326^+$ cells also expressed specifically cytokeratin-7 as proven by flow cytometry. CTs were then expanded in Amniomax serum-free medium containing commercially available supplements (Gibco, Karlsruhe, Germany). Cells from the unlabeled CD326-negative cellular fraction, referred to as VSCs, were also collected. VSCs were grown in DMEM (Gibco) containing 10% FCS (Gibco) and identified by flow cytometry using vimentin, a mesenchymal marker. CT and VSC cultures were typically used at passage 5.

$CD133^+$ cells (endothelial precursor cells) from human umbilical cord blood

Human umbilical cord blood (UCB) was collected from term pregnancies following caesarean section as previously described.[27] Briefly, the blood was diluted with PBS supplemented with 2 mM EDTA and subjected to density gradient centrifugation using Ficoll-Pacque (1.077 g/ml; Amersham Biosciences, Uppsala, Sweden). The mononuclear cell fraction was removed and CD133-expressing cells were isolated by using MACS employing an antibody against CD133. Owing to the coexpression of CD34, CD31 and CD133 antigens, these cells are also referred to as EPCs. EPC cultures were expanded in IMDM (Gibco) containing 10% FCS, 10 ng/ml stem cell factor, 20 ng/ml thrombopoietin and 50 ng/ml Flt-3-Ligand (all growth factors from Promokine, Heidelberg, Germany) for 7 days before the experiments were started. Medium was replaced every 3–4 days.

Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated based on established methods described by Jaffe *et al*[28] with minor alterations. In brief, the cord was rinsed with HBSS and filled with collagenase solution consisting of HBSS (Gibco) with Mg^{2+}/Ca^{2+} and 200–265 U/ml collagenase IV (Biochrom, Berlin, Germany). Both cord endings were pinched with forceps and the cord was incubated at 37°C for 20 min. The collagenase solution was collected, the vein washed with HBSS and digested endothelial cells were centrifuged at 1500 r.p.m. for 10 min. HUVECs were cultured in EBM-MV2 medium with supplied growth factors (PromoCell, Heidelberg, Germany) used up to passage 7.

Human placental trophoblast-conditioned medium

Conditioned media was produced by incubation of subconfluent CTs in IMDM without supplements for 12 h. Media were removed, centrifuged at 4000 r.p.m. for 10 min and stored at −80°C until required.

Formation of spheroids

For the investigation of cell–cell interactions between the isolated cellular components in vasculogenesis, a three-dimensional spheroid model was developed. To generate spheroids of defined size and cell number, 5×10^2 EPCs, 1.5×10^3 CTs and 1.5×10^3 VSCs (ratio 1:3:3) per spheroid were mixed and resuspended in cell culture medium containing AmnioMax™-100 (containing the appropriate commercially available supplements) and IMDM medium (ratio 1:1) supplemented with 0.25% (w/v) methyl cellulose and 1% FCS. The cell suspension was cultured in a 96-well plate (100 μl per well; Cellstar, Greiner Bio-One,

Frickenhausen, Germany) at 37°C in physiologically relevant oxygen concentrations as described below. Spheroid formation was observed within 18–20 h. Where applicable, EPCs and VSCs were labeled with a PKH-26 Kit (red) and a PKH2 Kit (green), respectively, as described by the manufacturer (Sigma, Munich, Germany). CTs remained unlabeled.

Spheroid Formation in Physiological Oxygen Environments

Spheroid formation was investigated in environments containing low oxygen thereby mimicking physiologically relevant oxygen concentrations as described previously and recently applied in our laboratory.[22,23] In brief, cells were prepared for spheroid formation as described above and incubated in Modular Incubator Chambers (Billups-Rothenburg, Del Mar, CA, USA) for 24 h. The chambers were flushed with appropriate gas mixtures for 4 min at a rate of approximately 25 l/min three times a day. The gas mixtures used in this study consisted of either 1 or 8% O_2. The modular incubator chambers were placed in a 37°C incubator during the course of the experiment. As a control, spheroids were cultivated in 21% O_2.

In vitro *invasion assay*

The *in vitro* invasion assay was conducted similarly to the recently described assays with minor modifications.[29,30] Spheroids were harvested, centrifuged at 1200 r.p.m., 4°C for 5 min and washed with PBS. Thereafter they were resuspended in 4 ml 1.2% (w/v) methyl cellulose in IMDM medium containing 1% FCS. A volume of 4 ml collagen (0.35 mg/ml collagen prepared from rat tails in 0.1% acetic acid) was supplemented with 400 μl 10 × 199-medium (Gibco) and neutralized with 500 μl 0.2 M NaOH. The pH value was adjusted to 7.4. The spheroids were mixed with collagen and equally distributed on a 24-well plate. The polymerization took place within 30 min at 37°C. Invasion was recorded under appropriate O_2 concentration using a live cell imaging system including a fluorescence microscope (Nikon TE2000; Nikon, Duesseldorf, Germany). Images were obtained every 15 min utilizing phase contrast, FITC and RhodRedX filters. Following a 24 h incubation period, live recording documentation of the experiments was generated using NIS Elements AR (version 2.3) software (Nikon).

Spheroid fixation and characterization

Spheroids were collected, fixed with 4% paraformaldehyde for 15 min, washed with PBS and resuspended in 100 μl collagen (0.35 mg/ml collagen in 0.1% acetic acid) for the embedding in Tissue-Tek®. Storage at −80°C and cryoslicing (8 μm) followed. The spheroids were transferred onto a thin layer of frozen TissueTek®, then overlaid with TissueTek® and stored at −80°C. Cryoslices were transferred to SuperFrost Ultra Plus object holders (Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany) and subjected to immunostaining as described below.

Immunocytochemistry

Cultures were centrifuged, washed with PBS and fixed in Zamboni solution containing paraformaldehyde and picric acid for 15 min at room temperature. Cells were washed in PBS overnight at 4°C and subsequently transferred to a SuperFrost Ultra Plus object holder to a final concentration of 1×10^6 per 10 μl and dried for 24 h. Fixed cells were washed in PBS, blocked with 10% donkey serum (PBS; 1% BSA, 0.1% Triton X-100) for 1 h and incubated with a primary antibody diluted in PBS supplemented with 1% donkey serum and 0.1% Triton X-100 for 1 h. Anti-CD68 (1:300) (BD, Heidelberg, Germany) and anti-VEGFR-3 (1:10) (R&D Wiesbaden-Nordenstadt, Germany) were used as primary antibodies. Thereafter, cells were washed, incubated in a 1:1000 dilution of Rhodamine Red-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) for 1 h and washed again. Nuclei were counterstained for 5 min with 5 μg/ml Hoechst 33342 (Merck Biosciences, Darmstadt, Germany). SuperFrost Ultra Plus object holders (Gerhard Menzel Glasbearbeitungswerk GmbH & Co.KG) were mounted with ProLong® Gold (Invitrogen, Karlsruhe, Germany). Fluorescent cells were visualized with a fluorescence microscope (Nikon TE2000; Nikon) and images captured using NIS Elements AR version 2.3 software (Nikon).

Immunohistochemistry of early placental tissue and spheroids

Early placental tissue (5 × 5 × 5 mm pieces) and spheroids were collected, fixed with HOPE®-Fixation (DCS, Hamburg, Germany) and paraformaldehyde (4%), respectively, embedded in Tissue-Tek® OTC (Sakura Finetek Europe, Zoeterwoude, Holland) and immediately immersed in liquid nitrogen. Frozen blocks were stored at −80°C until cryoslicing (8 μm). Slices were blocked as described above and incubated with a primary antibody overnight at 4°C. Antibodies used were anti-cytokeratin-7 (1:40) (Dako, Hamburg, Germany), anti-vimentin (1:100) (Santa Cruz, Heidelberg, Germany), anti-CD31 (1:50) (Dako), anti-SDF1-α (Santa Cruz) (1:50), anti-CXCR4 (1:100) and anti-CD34 (1:100) (Immunotech, Hamburg, Germany). The secondary Alexa 488- or Alexa 555-conjugated antibody (1:1500) (BD) was applied and nuclei were stained as mentioned above. ProLong® Gold (Invitrogen) mounting and visualization with the fluorescence confocal microscope followed.

Tube formation assay

The ability of endothelial cells to form tubes or networks was tested as previously described.[22] In brief, growth factor-reduced Matrigel™ was diluted 1:2 with cold IMDM without supplements and pipetted in volumes of 50 μl per well in a 96-well plate (150–200 μl per 1 cm^2) and polymerized 1–2 h at 37°C. Subsequently, 1×10^4 HUVEC- and 1×10^4 PKH-26-labeled EPCs were mixed, then transferred to the matrix where capillary net structures formed within 20 h. Net structures were visualized with a fluorescence microscope (Nikon) and images captured using NIS Elements AR version 2.3 software (Nikon).

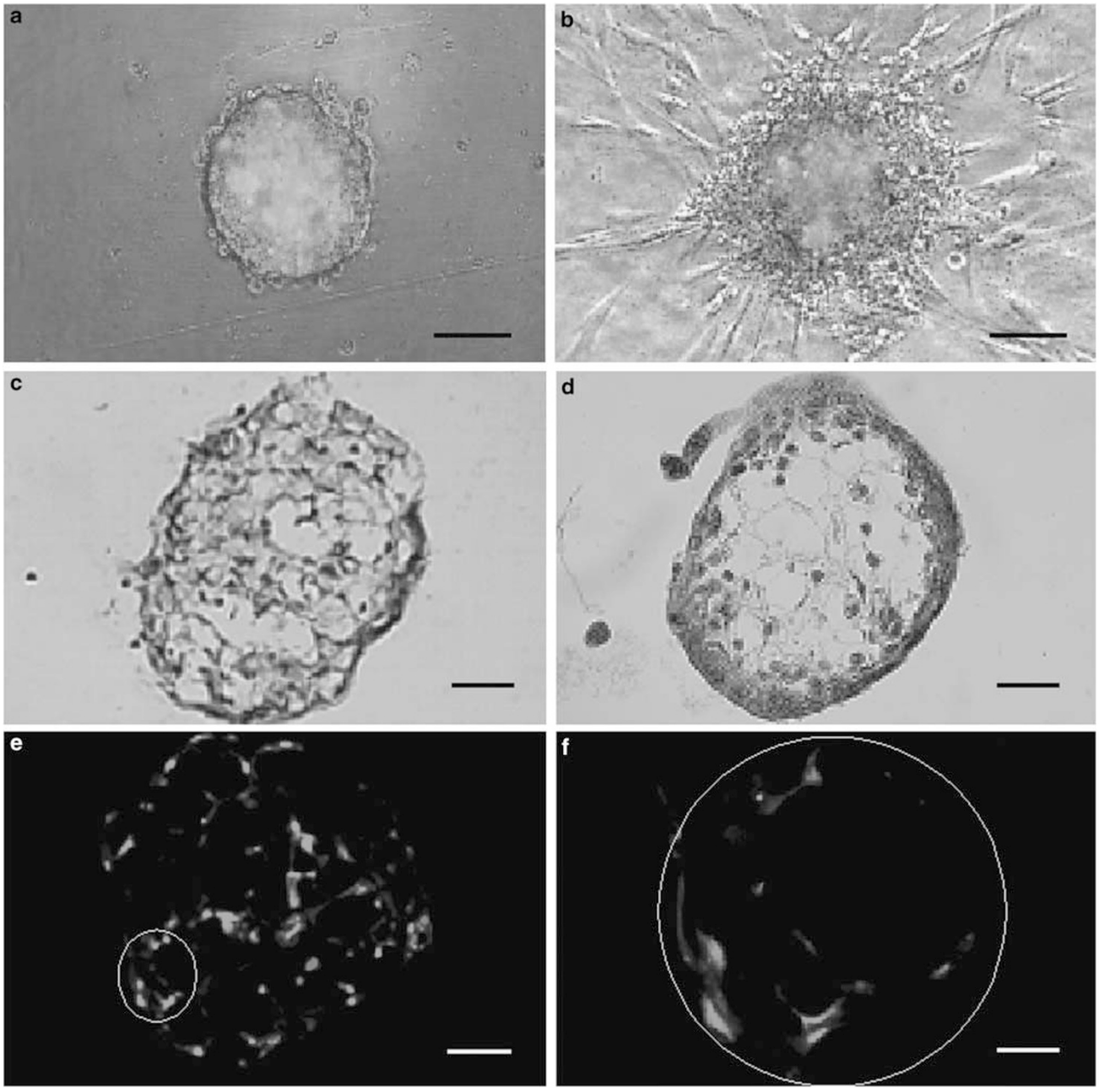

Figure 1 A three-dimensional spheroid composed by EPCs, VSCs and CT. (**a**) EPCs and VSCs were fluorescently labeled (red and green, respectively) and subsequently cocultured with unlabeled CTs. After approximately 20 h, spontaneous formation of spheroids could be observed. Bar 150 μm. (**b**) The spheroids were embedded in a gel-like collagen matrix and incubated for 24 h. CTs leaving the spheroidal core show the invasive phenotype. Bar 150 μm. (**c**) The spheroids were collected, fixed with PFA and embedded in a collagen matrix which was cryoconserved in Tissue-Tek® before cryoslicing (8 μm). Slices were subjected to hematoxylin and eosin staining, and the inner structure could be identified as a network formed by CTs, VSCs and EPCs. Bar 50 μm. (**d**) Early placental villi were also cryoconserved in Tissue-Tek® and hematoxylin and eosin stained following cryoslicing (8 μm). The cross-sections of the spheroid and placental villi show similar structures. Bar 50 μm. (**e**, **f**) PKH staining of EPCs (red) and CTs (green) within a spheroid. Bars 50 and 10 μm, respectively.

RESULTS

Spheroid Development and Characterization

Following set up of the coculture system, spheroid formation was accomplished in approximately 18 h and resulted in an average diameter of 200 μm per spheroid (Figure 1a). Cross-sections of H&E-stained spheroids (Figure 1c) and early placental villi (Figure 1d) revealed significant structural similarities between both systems. Further characterization of spheroids was accomplished by fluorescence-microscopic analysis of cross-sections (Figure 1e and f).

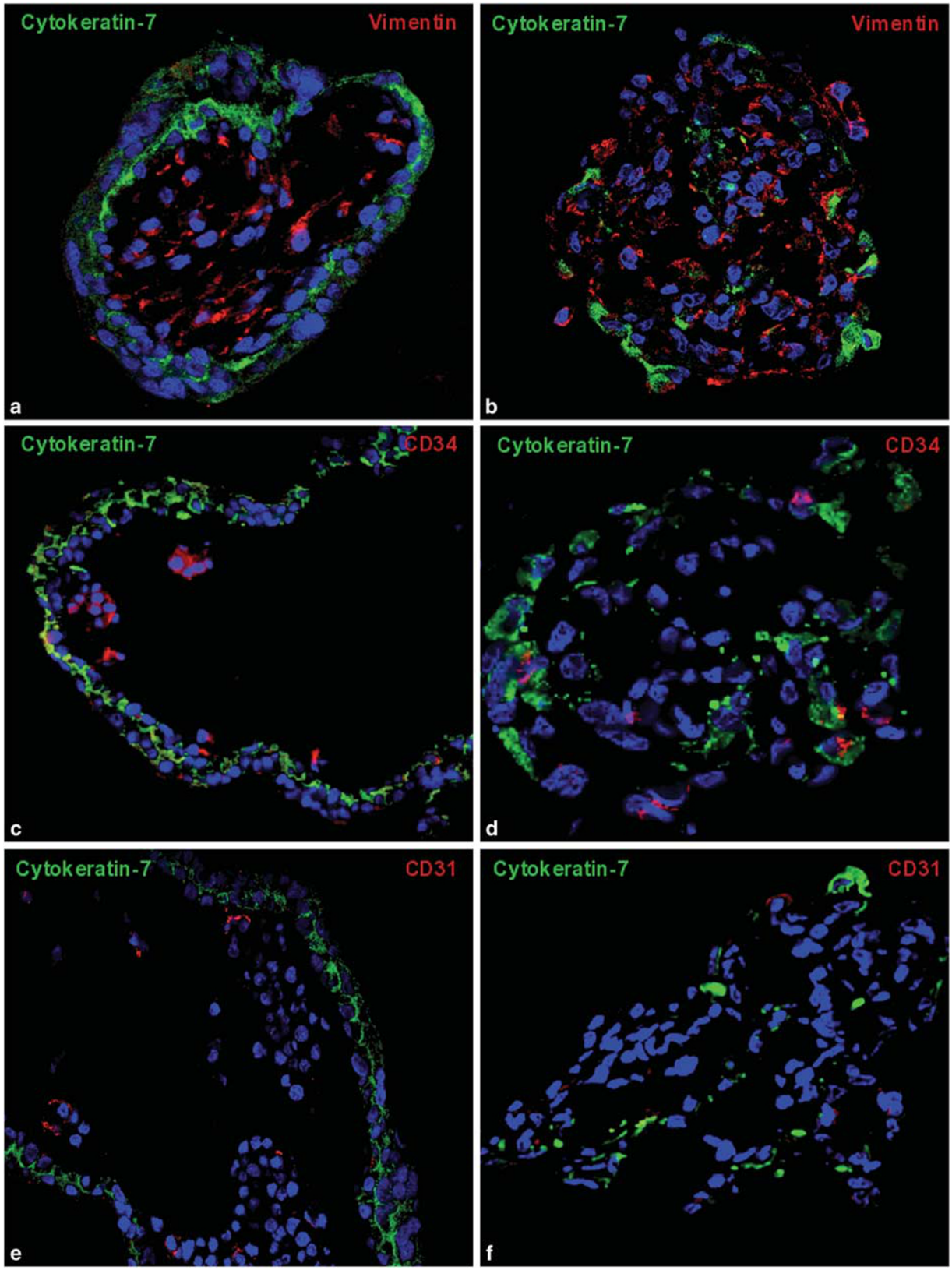

Figure 2 Immunohistochemical analysis of human placental villous tissue and spheroids. (**a**–**f**) Differentiation patterns of spheroids comprising CTs, VSCs and EPCs correlate to tissues from early placenta (7 w.p.c.) as shown by immunohistochemical staining. (**a**, **b**) CTs were labeled with anti-cytokeratin-7 (green), VSCs with vimentin antibodies (red). (**c**, **d**) CD34 expressed on EPCs stained with human anti-CD34 (red). (**e**, **f**) CD31 expressed on EPCs stained with human anti-CD31 (red). Cell nuclei were stained with Hoechst (Blue).

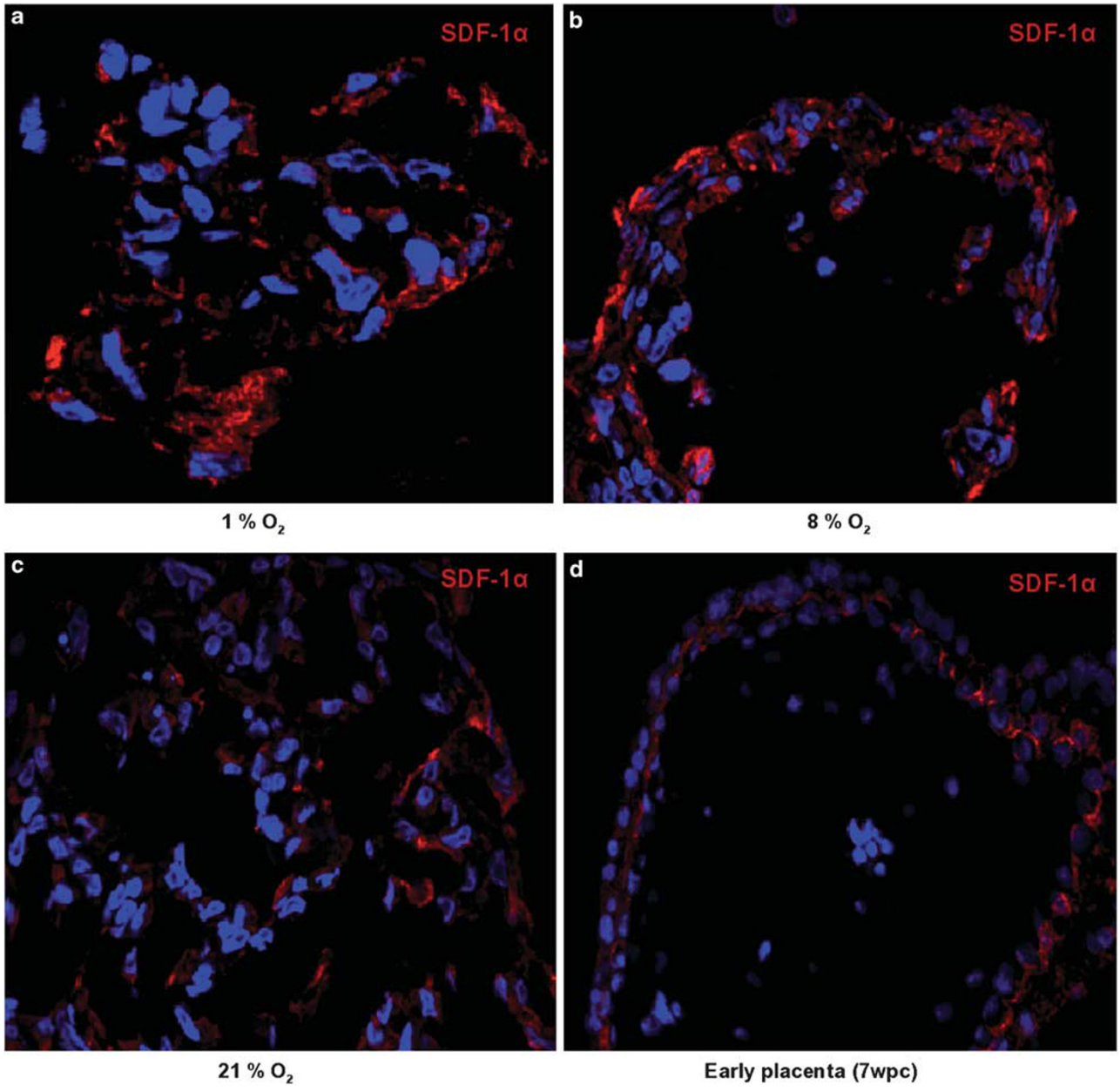

Figure 3 Spheroids show enhanced expression of SDF-1α in a hypoxic environment. Immunohistochemistry was utilized to examine spheroidal SDF-1α protein expression in (**a**) 1% O_2, (**b**) 8% O_2, (**c**) 21% O_2 and (**d**) in human placental tissue from 7 w.p.c.. Tissues were immunolabeled with anti-human SDF-1α (red) and nuclei stained with Hoechst (Blue).

Spheroids were prepared for *in vitro* invasion assays, by embedding in a collagen matrix followed by incubation for 24 h. CTs could be observed invading radially out of the spheroid outer layer into the collagen matrix (Figure 1b).

Subsequent immunhistochemical analysis of sections from early placental villi (Figure 2, left panels) and spheroids (Figure 2, right panels), respectively, were performed. Tissues and spheroids were probed with specific fluorochrome-coupled antibodies against human cytokeratin-7, vimentin, CD34 and CD31. Cell nuclei were counterstained with Hoechst (Blue). Remarkable similarities between placental villi and spheroids are shown in Figure 2a–f. Cytokeratin-7-expressing CT were organized in a characteristically fashion around the villous core. Vimentin-expressing cells were located in the center of the spheroid and the villus from early placenta (Figure 2a and b). A significant number of hematopoietic and EPCs ($CD34^+$ cells) were detected within the spheroidal core and the villous stroma (Figure 2c and d). Differentiated endothelial cells ($CD31^+$) were faintly detected in focal areas of the spheroid and villous tissue (Figure 2e and f).

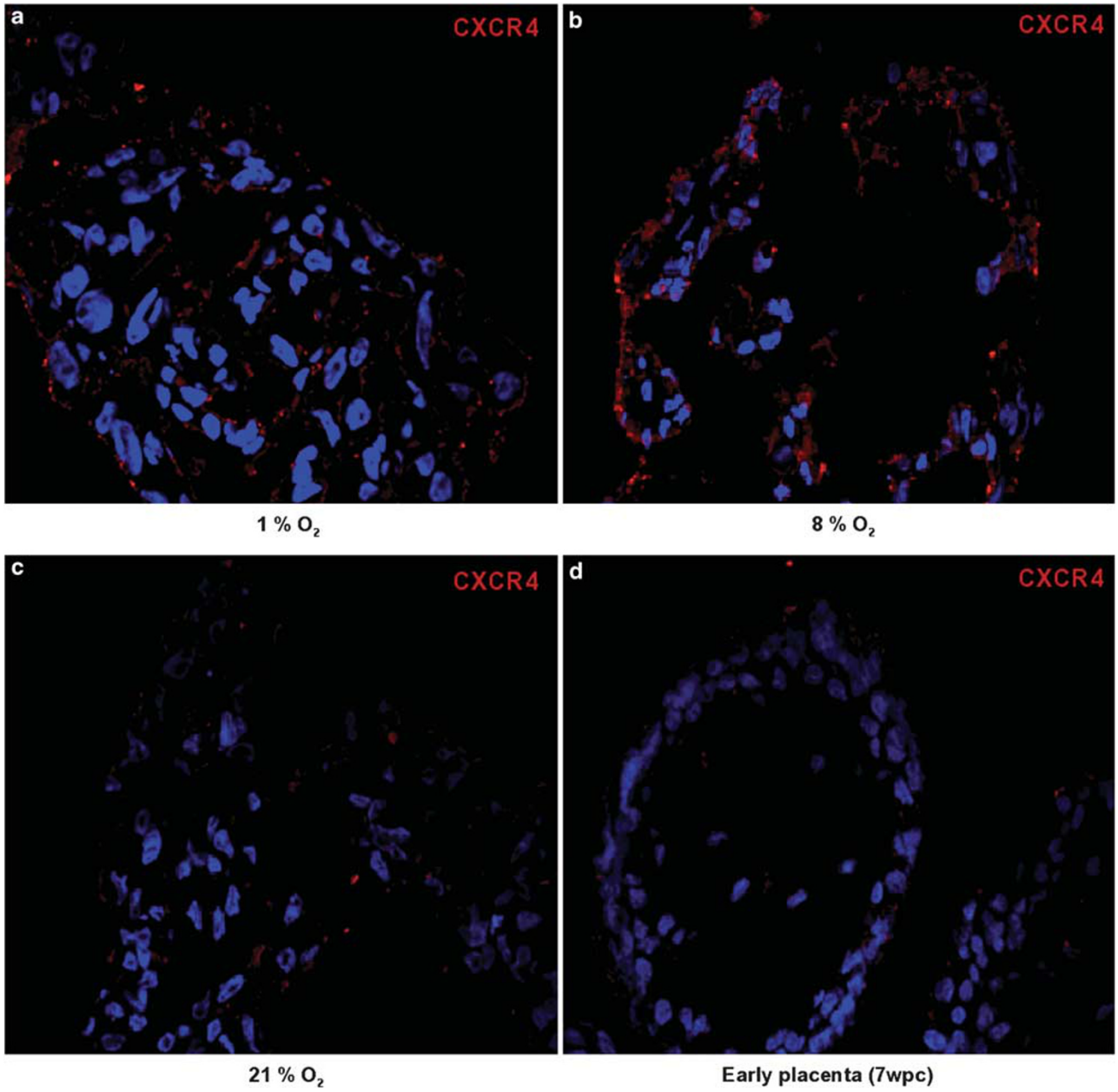

Figure 4 Spheroids show enhanced expression of CXCR4 in a hypoxic environment. Immunohistochemistry was utilized to examine spheroidal CXCR4 protein expression in (**a**) 1% O_2, (**b**) 8% O_2, (**c**) 21% O_2 and (**d**) in human placental tissue from 7 w.p.c. Tissues and speroids were immunolabeled with anti-human SDF-1α (red) and nuclei stained with Hoechst (Blue).

Enhanced SDF-1α- and CXCR4 Expression in Spheroids Under Low Oxygen

Immunohistochemistry was employed to test the expression of SDF-1α and the corresponding receptor CXCR4 following 24 h incubation of spheroids under hyperoxic (21%) and hypoxic (8 and 1%) conditions. When spheroids were incubated in 8% O_2, a mean pO_2 of 65.0 ± 7.5 mm Hg was maintained. This environmental condition is similar to the oxygen tension found in placental beds from pregnancies at 10–15 w.p.c. (65.0 ± 3.4 mm Hg).[31] When spheroids were incubated in 1% O_2, the mean pO_2 measured was 10.2 ± 5.5 mm Hg. These oxygen tensions were similar to previously described physiological pO2 in the placenta at 8–10 w.p.c. (17.9 ± 6.9 mm Hg).[23] SDF-1α- and CXCR4 immunoreactivity was detected in early tissue as well as in spheroids (Figure 3). If cultured in low-oxygen concentrations (1 and 8% O_2), enhanced SDF-1α expression in spheroids was noted (Figure 3a and b) as compared to 21% O_2 (Figure 3c and d). In Figure 4, CXCR4 immunoreactivity in low oxygen and hyperoxic environments is illustrated, whereby CXCR4 expression in low-oxygen concentrations is enhanced (Figure 4a and b) as compared to 21% O_2 (Figure 4c and d).

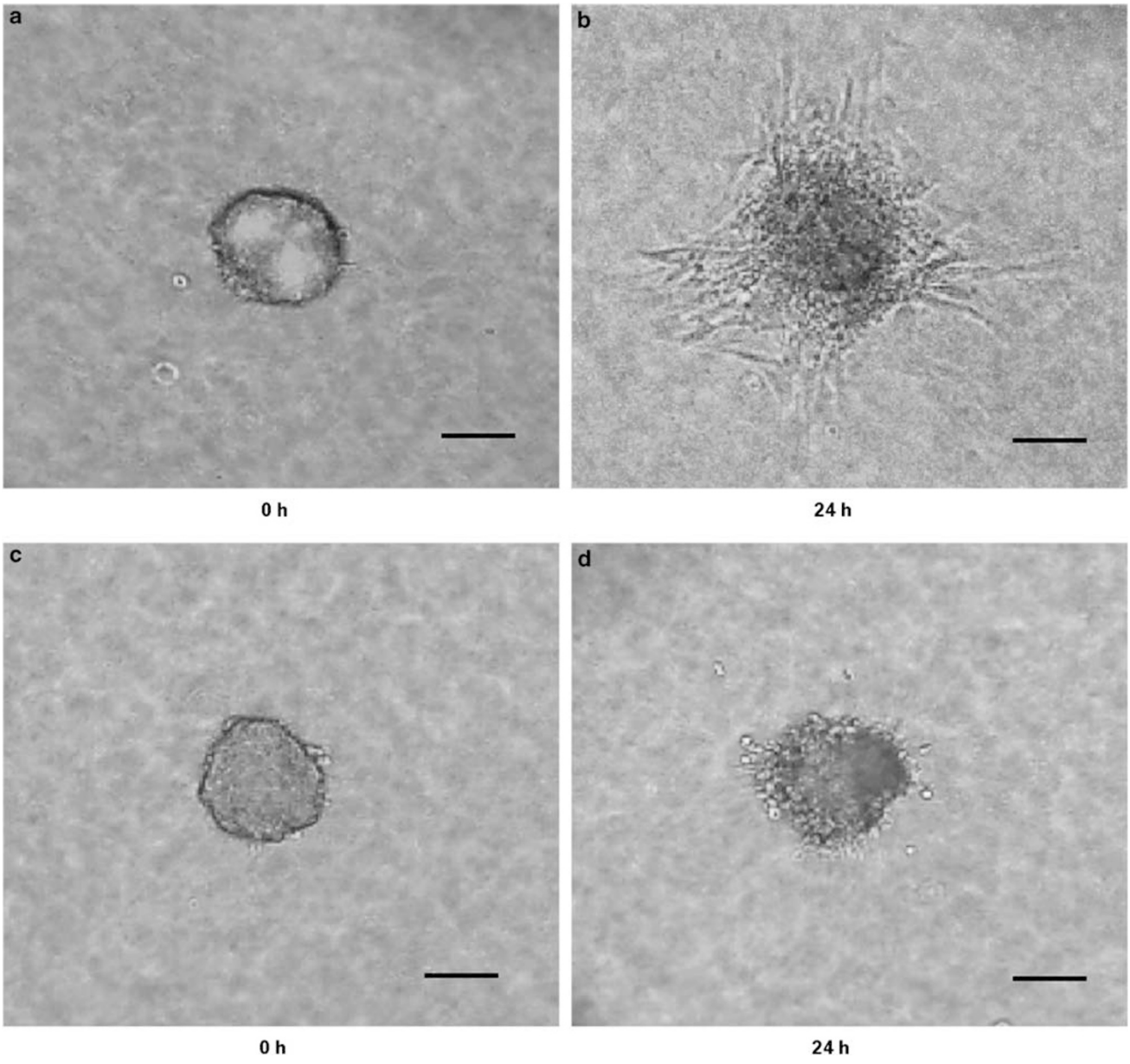

Figure 5 Hypoxia-induced CT invasion. (**a**, **b**) Invasion into a collagen matrix induced by incubation in a physiologically low-oxygen environment (1% O_2) during the course of the experiment (24 h). (**c**, **d**) Incubation in atmospheric oxygen (21% O_2) showed reduced invasion. Bars 150 μm.

CT Invasion in Physiologically Relevant Oxygen Tensions

To study CT invasion out of the spheroids, an *in vitro* invasion assay during additional 24 h incubation under appropriate oxygen concentrations was applied. During the course of the experiment, *in vitro* invasion was monitored using a live cell imaging system. CT invaded into the collagen matrix under the hypoxic environment (Figure 5a and b; Supplementary Video 1) which only scarcely proceeded under 21% O_2 (Figure 5c and d; Supplementary Video 2).

DISCUSSION

In previously published *in vitro* studies of placental vascular development, different types of placental cells formed a two-dimensional mononuclear layer supposedly mimicking many of the phenotypic and functional properties in the villous tissue.[24,32] Unfortunately, these two-dimensional cell culture systems have significant limitations as cells may lose their functional and differentiated phenotypic properties. For example, endothelial cell differentiation antigens such as CD34 are downregulated after transfer into two-dimensional culture system, and other endothelial cells lose their

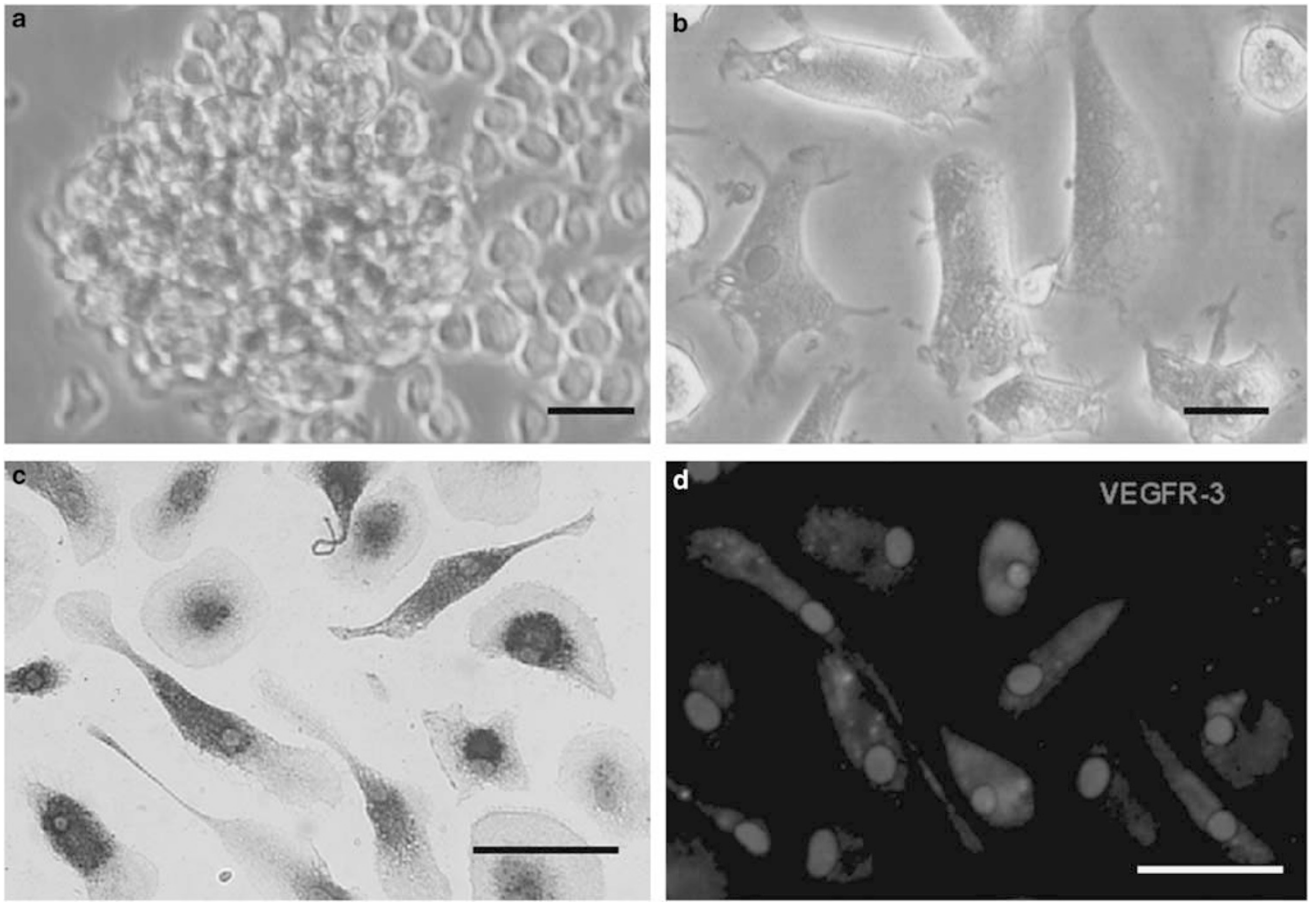

Figure 6 Morphology of long-term-cultured EPCs in HTR-CM. EPCs were cultured in HTR-CM supplemented with 1% FCS. (**a**) After 4 weeks incubation, cells formed spheroids that did not attach to the bottom of the culture flask. Bar 20 μm. (**b**) After following 24 h incubation in IMDM medium supplemented with 1% FCS, cells became adhesive and showed an endothelial-like morphology (bar 20 μm), expressed macrophagial CD68 (**c**) and VEGFR-3 (**d**) after 24 h incubation in IMDM medium supplemented with 1% FCS following 4-week incubation in HTR-CM. Nuclei are counterstained with Hoechst (Blue). Bars 50 μm.

tight junction-dependent characteristics in two-dimensional culture.[33,34]

Instead of two-dimensional cell culture systems, spheroid models of tumor- and embryonic stem cells have been extensively employed to investigate cellular differentiation, cell–cell interactions, cellular hypoxia responses as well as in therapeutically oriented studies.[35–39] Recently established three-dimensional endothelial spheroid models of vascular differentiation have shown a positive influence on cellular apoptosis, cell–cell signaling and expression of vasoactive cytokines and growth factors.[40–43]

To investigate distinct differentiation patterns in the placenta, we developed a coculture model including three major placental cell types. Aggregates of placental-derived EPCs, CTs and VSCs were generated as described above. Similar techniques have recently been applied to generate spheroid cultures of CT and endothelial cells.[40,44] The aggregates developed spontaneously in nonadherent culture plates and resulted in stable spheroids. In our model, the placental spheroids revealed significant structural similarities with the differentiation pattern of early placental tissue. Similar to isolated placental villi, differentiation antigens are expressed on CTs, VSCs and EPCs in the new spheroidal model. Cytokeratin-7, a specific marker for CT cells, stains around the spheroidal core and resembles *in vivo* villous architecture. However, this spheroid model failed to form syncytiotrophoblasts, which could not be detected by immunostaining with NDOG1, a syncytiotrophoblast-specific antibody. In human placenta, syncytiotrophoblasts reveal multinucleated structures resulting from a fusion of mononuclear CTs.[8] Therefore, extention of the incubation time for spheroid formation may be required in order to detect cytotrophoblastic cell fusions.

It was further demonstrated that spheroids recruited $CD34^+$ cells similar to the chorionic villous stroma from early placental tissue. Although the utilization of UCB-derived EPCs derived from term placenta may represent a disadvantage of this coculture system, there is no alternative for $CD133+$ cells coexpressing the endothelial marker CD34 from UCB at present.[45] Although addition

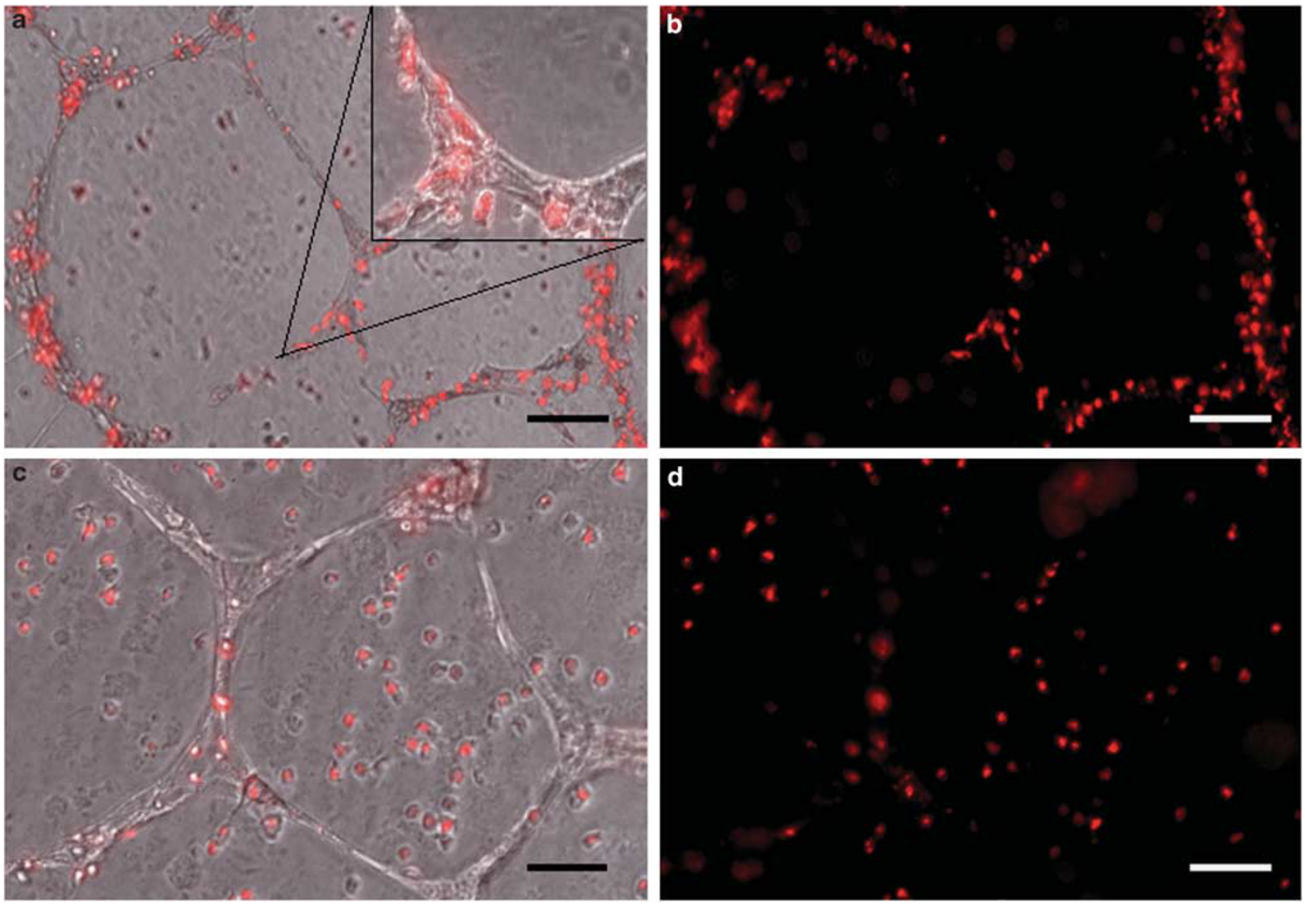

Figure 7 Endothelial cell network formation. Fluorescently labeled EPCs were cultivated in HTR-CM supplemented with 1% FCS for 4 weeks and subsequently cocultured with HUVECs on growth factor-reduced Matrigel. (**a**) Phase contrast micrograph overlaid with fluorescent micrograph: EPCs were incorporated into the network formed by HUVEC after 14 h; (**b**) fluorescent micrograph of incorporated EPCs. (**c**) Phase contrast micrograph overlaid with fluorescent micrograph: EPCs incubated in IMDM medium supplemented with growth factors and 10% FCS before cocultivation with HUVECs did not incorporate into the HUVEC network; (**d**) fluorescent micrograph of not incorporated EPCs. Bars 150 μm.

of progenitor cells from first trimester placental tissue would be more appropriate, the number of isolated cells would not be sufficient for spheroid generation. Another disadvantage of our model is the inherent difficulty in quantifying antigen expression. To solve this problem in future studies, it may be appropriate to dissociate cellular components from the spheroid and subsequently employ flow cytometry or cell picking methods. Also, it may be of some interest to investigate immunophenotyping after transferring cellular components back to monolayer cultures as recently reported by Potapova *et al*.[46]

Despite the disadvantages described above, the spheroid model described here offers a whole spectrum of opportunities to study both, physiological as well as pathological events in the early placenta. For example, the spheroid model may be relevant for testing the influence of toxins or viral exposure on vascular development. Recently, a three-dimensional spheroid assay to measure viral-induced oncolysis was successfully established.[47] The infection of spheroids with different viruses may be used in studies on envelope genes from endogenous retroviruses termed syncytins specifically expressed in the placenta.[48]

Multiple models based on two-dimensional monolayer and explant cultures have been established for analysis of trophoblast differentiation and invasion.[49–55] Korff *et al*[44] successfully developed an invasion assay using a spheroidal CT model to mimic the *in vivo* phenotype and differentiation of first trimester, third trimester and preeclamptic CT. Our assay combined with the application of the Modular Incubator Chamber allows the investigation of CT invasion and differentiation under low-oxygen environments. CTs leaving the spheroidal core are indicative of the invasive phenotype. These findings are in concert with previously published observations. First trimester CTs intensively invaded the collagen-gel whereas normal and preeclamptic CT cells from third trimester, when maternal blood flow and oxygen supply is fully established, showed a much lower invasive capacity.[44] In addition, we were able to demonstrate that the expression of SDF-1α/CXCR4 system crucial for

migration and adhesion of precursor and stem cells was regulated by oxygen. Our data are in concert with previously published results showing that mRNA- and protein expression of SDF-1α and CXCR4 is regulated in synovial fibroblasts in a low-oxygen environment.[56] Furthermore, the recruitment of CXCR4-positive progenitor cells to regenerating ischemic tissues is mediated by hypoxic gradients via HIF-1-induced expression of SDF-1 as showed by Ceradini *et al*.[57]

As cell–cell interactions are important in differentiation of progenitor and stem cells, our model provides additional insights into these processes during placental vascular development. EPCs grown in human placental trophoblast-conditioned medium (HTR-CM) interacted and formed multiple cell spheroids that did not adhere (Figure 6a). They became adhesive, showed an endothelial-like morphology (Figure 6b) and stained with CD68 and VEGFR-3 after the transfer into IMDM medium (Figure 6c and d). We were also able to show that EPCs in HTR-CM were incorporated into capillary networks of endothelial cells (Figure 7a–d).

In conclusion, the three-dimensional spheroid model described in this investigation has proven to be an adequate system for investigations regarding the *in vivo* situation of placental vasculogenesis and trophoblast invasion. The integration of CTs, VSCs and EPCs into one spheroid, a structure that is morphologically similar to placental villi, will be applicable for future experiments. This spheroid model also allows cultivation of cells in close contact to each other in physiologically relevant low-oxygen environments, further mimicking the *in vivo* environment.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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