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Human Endothelial Cells Inhibit BMSC Differentiation Into Mature Osteoblasts In Vitro by Interfering With Osterix Expression

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Abstract It is well accepted, that there is communication between osteoblasts and endothelial cells. However, the influence of endothelial cells on the differentiation of bone precursors into mature osteoblasts is not yet well understood. We therefore studied the effect of human umbilical vein endothelial cells (HUVEC) on human bone marrow stromal cell (BMSC) differentiation towards an osteoblastic phenotype by culturing them in two different types of HUVEC-BMSC cultures (indirect contact, HUVEC-conditioned medium). Typical bone markers (^{45}Ca incorporation, ALP activity, and gene expression of collagen 1 (COL1), osteonectin (ON), matrix metalloproteinase 13 (MMP-13), bone morphogenetic protein 2 (BMP-2)) and transcription factors (Runx2, osterix (OSX)) were evaluated at different time points. The effect of stimulating HUVEC with vascular endothelial growth factor (VEGF) before co-cultures with BMSC was also evaluated. As expected, BMSC in osteogenic medium (OM) (with dexamethasone) differentiated towards the osteoblastic phenotype, as measured by increased matrix mineralization, high ALP activity, and elevated expression of specific osteoblastic marker genes, when compared to BMSC in non-OM. HUVEC reversibly inhibited osteoblastic differentiation of BMSC in OM, independent of the co-culture type. When HUVEC were removed from the cultures, BMSC differentiated into osteoblasts, albeit at a faster rate than BMSC that were never co-cultured with HUVEC. Stimulation of HUVEC with VEGF before co-culture enhanced the inhibitory effect of HUVEC on BMSC differentiation. This inhibitory effect was connected to a reversible suppression of OSX gene expression and was specific to endothelial cells. We conclude that HUVEC can inhibit dexamethasone-induced BMSC differentiation into osteoblasts in vitro, by interfering with OSX expression, thereby arresting BMSC differentiation at a preosteoblastic stage. J. Cell. Biochem. 98: 992–1006, 2006. © 2006 Wiley-Liss, Inc.

Key words: bone marrow stromal cells; dexamethasone; vascular endothelial cells; differentiation; osterix

Bone marrow stromal cells (BMSC) derive from the adherent, mononucleated fraction of bone marrow [Friedenstein, 1995]. It has been shown that this cell pool contains precursors for several mesenchymal tissue cells including osteoblasts, fibroblasts, chondrocytes, adipocytes, and myoblasts [M Owen, 1988; ME Owen, 1998; Pittenger et al., 1999]. Bone marrow stroma is actually suggested to be the main source for osteoblastic precursors [M Owen, 1988]. Recruitment, proliferation, and differentiation of BMSC into mature osteoblasts are regulated by many factors including cytokines, systemic hormones, growth factors, and other regulators [Lian and Stein, 1995]. These factors are not only released by the osteoblastic cells themselves, but also by cells that are part of the tightly connected vascular system, such as endothelial cells [Wang et al., 1997; Villars et al., 2000; Street et al., 2002] or pericytes [Jones et al., 1995]. Endothelial precursors can be isolated from bone marrow, as well as from peripheral blood [Watt et al., 1995; Peichev et al., 2000]. During embryonic development, the major vascular network is formed by vasculogenesis, mediated mainly by factors belonging to the vascular endothelial growth factor (VEGF) family [Risau, 1997]. VEGFs bind to several receptors including Flt-1 (VEGFR-1) and KDR (VEGFR-2 or Flk-1) that have been identified on many cells types, including endothelial and osteoblastic cells [Horner et al., 2001; Moyon et al., 2001; Deckers et al., 2002]. VEGFs promote vascular endothelial cell proliferation and induce vascular leak and permeability, which allows the initial network to be

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remodeled by angiogenic sprouting [Ferrara, 1999a,b], a process in which the immature and poorly functional vascular network is remodeled into a complex network of mature and stable blood vessels [Folkman and D'Amore, 1996]. It is widely accepted that there must be communication between endothelial cells and osteoblastic cells in order to regulate blood vessel formation, osteoblast differentiation, and bone turnover, processes that are all very tightly connected [Pechak et al., 1986; Brighton, 1987; Brighton and Hunt, 1991; Decker et al., 1995; Chang et al., 1996; Bittner et al., 1998]. In vivo and in vitro experiments suggest a paracrine communication between endothelial cells and osteoblastic cells using gap junctions to mediate the angiogenic process required for bone formation and repair [Villars et al., 2002]. VEGF is believed to play an important role in these processes [Guenther et al., 1986b; Villanueva and Nimni, 1990; Jones et al., 1995; Goad et al., 1996; Schlaeppi et al., 1997; Wang et al., 1997]. It was shown, in a mouse model, that angiogenesis, bone formation, and callus mineralization in femoral fractures do not occur, if VEGF activity is inhibited [Street et al., 2002]. In vitro studies have shown that VEGF has a stimulatory effect on osteoblastic differentiation [Deckers et al., 2000]; however, it does not seem to have a direct effect on osteoblastic precursor cell proliferation [Villars et al., 2000; Furumatsu et al., 2003]. VEGF is definitely not the only factor involved in these communication processes. There are several other factors that are expressed and secreted during bone healing and that can affect osteoblastic cells or endothelial cells, including PDGFs, TGFs, FGFs, angiopoietins, and endothelins [Gross et al., 1983; Guenther et al., 1986a; Gittens and Uludag, 2001; Mandracchia et al., 2001].

In vitro studies about the influence of endothelial cells on osteoblastic differentiation have shown a remarkable variety of results [Jones et al., 1995; Wang et al., 1997; Villars et al., 2000]. These differing results are partly due to the different cell (lines) and culture conditions used as well as due to different donor age and biopsy site. In addition, we hypothesize that the differentiation state of endothelial cells, following stimulation with different factors, could play an important role on how they influence BMSC differentiation. In the present study, we have grown endothelial cells with or without VEGF stimulation and investigated their effect on BMSC differentiation towards the expression of an osteogenic phenotype.

MATERIALS AND METHODS

Origins of Human Cells

Bone marrow aspirates were taken from patients undergoing routine orthopedic surgery involving iliac crest exposure, after informed consent. Bone marrow aspirates (20 ml) were harvested into CPDA-containing Sarstedt monovettes using a biopsy needle that was pushed through the cortical bone. Aspirates were processed within 12–24 h after harvesting.

Isolation and Expansion of Human BMSC

Bone marrow aspirates were homogenized by pushing them a few times through a syringe. The aspirate was then diluted 1:4 with IMDM (Gibco 42200-022) containing 5% (v/v) FBS (Gibco 10270-106) and centrifuged at 200*g* for 5 min at room temperature (RT). The top layer (approx. 1 cm) containing mostly fat tissue was removed. Per 1 ml of undiluted sample, 2.6 ml of Ficoll (Histopaque-1077, Sigma #1077-1) was pipetted into a 50-ml Falcon tube and the aspirate was added carefully on top of the Ficoll. After centrifugation at 800*g* for 20 min at RT, the mononucleated cells were collected at the interphase using a syringe. To 1 ml of collected interphase solution, 5 ml of IMDM/5% FBS was added, the tube was gently mixed and centrifuged at 400*g* for 15 min at RT. The pellet was resuspended in the same amount of IMDM/5% FBS, centrifuged again, and resuspended in IMDM/5% FBS. Cell number was determined using Methylene Blue in a hemocytometer. The cells were seeded at densities of $8–10 \times 10^6$ mononucleated cells per 150 cm^2 T-flask in IMDM containing 10% FBS, nonessential amino acids (Gibco 11140-035) and PenStrep (100 U/ml, Gibco 15140-122). After 5 days, the monolayers were washed with Tyrode's balanced salt solution (TBSS) to remove non-adherent cells, and fresh medium containing 5 ng/ml FGF-2 (R&D 233-FB) was added as previously reported [Martin et al., 1997; Muraglia et al., 2000; Bianchi et al., 2003]. Medium was changed every 2–3 days and cells were subcultured 1:3 at subconfluence. The adherent cells after one subculture were termed BMSC. Only cells between passages 2–4 (approx. 12–18 populations doublings) were subsequently used [Banfi et al., 2000].

Expansion of Human Umbilical Vein Endothelial Cells (HUVEC)

Primary HUVEC were obtained from Cascade Biologics (cat# C-003-5C). The cells were expanded in IMDM containing 10% FBS, non-essential amino acids, PenStrep, 20 μg/ml ECGS (Sigma E-2759), and 90 μg/ml heparin (Sigma H-3149). Only cells between passages 2–4 were subsequently used.

Stimulation of HUVEC With VEGF

Human umbilical vein endothelial cells were expanded in IMDM containing 10% FBS, non-essential amino acids, 20 μg/ml ECGS, 90 μg/ml heparin, and PenStrep. At about 75% of confluency, 25 ng/ml VEGF (Sigma V-7259) was added and the cells were stimulated for 3 days [Street et al., 2002]. The HUVEC monolayers were then either used to produce HUVEC-conditioned medium [see Conditioned Medium (VEGF Stimulated or Non-Stimulated) on BMSC Cultures] or were trypsinized and used for indirect contact co-cultures.

BMSC Cultures

Bone marrow stromal cells were seeded in 24-well plates (Falcon BD 353504) at densities of 10,000–30,000 cells/well in 1 ml of medium and were left to attach for 2–3 h. Culture medium for the BMSC culture systems contained IMDM, 10% FBS, nonessential amino acids, 0.1 mM ascorbic acid-2-phosphate (Sigma A-8960), and 10 mM β-glycerophosphate (Sigma G-6251) with 10 nM dexamethasone [osteogenic medium (OM)] or without dexamethasone (non-OM). Dexamethasone was purchased at Sigma (D-2915). Media were changed twice a week.

Conditioned Medium (VEGF Stimulated or Non-Stimulated) on BMSC Cultures

Human umbilical vein endothelial cells conditioned medium was produced by washing the VEGF-stimulated (see Stimulation of HUVEC With VEGF) or non-stimulated HUVEC monolayers 2× with phosphate buffered saline (PBS) and then culturing them for 24 h in IMDM containing 10% FBS and PenStrep. After 24 h, the medium (defined as VEGF-stimulated or non-stimulated HUVEC-conditioned medium) was removed, filtered (0.22 μm), and stored at −20°C until used. This HUVEC-conditioned medium was added in a 1:10 mixture to BMSC monolayers cultured in OM or non-OM in 24-well plates. Chondrocyte-conditioned medium (Chondro-CM) using primary bovine chondrocytes and fibroblast-conditioned medium (Fibro-CM) using the human hTERT-BJ1 fibroblast cell line were also similarly prepared and applied accordingly.

Indirect Contact Cultures of BMSC and HUVEC (VEGF Stimulated or Non-Stimulated)

To BMSC monolayers, VEGF-stimulated (see Stimulation of HUVEC With VEGF) or non-stimulated HUVEC were added in cell culture inserts (0.4 μm pore size, Falcon BD 353495) at a density of 10,000 cells/insert. Fresh HUVEC were added once a week to the system to provide a fresh supply of endothelial cell secreted factors. These experiments were also performed in OM or non-OM.

Recovery Experiment

BMSC were grown in OM (or non-OM) supplemented 1:10 with non-stimulated HUVEC-conditioned medium, as described above. At different time points, medium supplementation with HUVEC-conditioned medium (non-stimulated) was stopped, and the BMSC were grown in OM (or non-OM). On day 27, the osteogenic differentiation stage of BMSC was evaluated by determining matrix mineralization and osterix (OSX) gene expression.

Inhibition Experiment

BMSC were grown in OM (non-OM as negative control). At different time points, the medium was switched to OM containing non-stimulated HUVEC-conditioned medium (1:10). After 28 days, the osteogenic differentiation was evaluated by determining ALP activity and OSX gene expression.

DNA Quantification

The protocol used for DNA quantification was based on the method described by Labarca and Paigen [1980], which involves the binding of Hoechst 33258 to the minor groove of the DNA double helix resulting in a measurable enhancement in fluorescence. BMSC were digested by adding 0.5 ml proteinase K (0.5 mg/ml proteinase K in phosphate buffer containing 3.36 mg/ml disodium-EDTA) solution directly to the wells and incubating the plates at 56°C for 1 h. Thereafter, the digested cells were transferred to Eppendorf tubes to avoid evaporation and were further digested overnight at 56°C.

After appropriate dilution with Dulbecco's phosphate buffered saline (DPBS) containing 0.1% (v/v) H33258 (from 1 mg/ml stock, Polysciences, Inc., 09460), the samples were measured using a PE HTS 7000 Bio Assay Reader at 360 nm excitation and 465 nm emission wavelength.

Quantification of Matrix Mineralization Using $^{45}Ca^{2+}$ Isotope

1.25 μCi/ml of $^{45}Ca^{2+}$ isotope (Amersham CES3) was added to each well and the plates were incubated at 37°C for 6 h [Alini et al., 1994]. The medium was removed and the monolayers were washed three times with IMDM to remove unincorporated $^{45}Ca^{2+}$. Then 0.5 ml of 70% formic acid was added to each well and the plates were incubated at 65°C for 1 h. The formic acid solution was then transferred to 3.5 ml of scintillation liquid (OptiPhase HiSafe'3 by Perkin Elmer) and the amount of radioactivity was measured using a Wallac 1414 WinSpectral liquid scintillation counter.

Quantification of Alkaline Phosphatase Activity

The medium was completely removed and the monolayers were washed once with PBS. The cell layers were extracted by addition of 500 μl of 0.1% Triton-X in 10 mM Tris-HCl (pH 7.4) and incubation at 4°C on a gyratory shaker for 2 h (See Sigma Technical Bulletin Procedure No.104). ALP activity was measured colorimetrically by measuring the *p*-nitrophenol production during 15 min incubation at 37°C with *p*-nitrophenyl phosphate as substrate (Sigma Kit No.104) on a Perkin Elmer Bio Assay Reader HTS 7000.

Quantification of VEGF Amounts in the Culture Supernatant

Culture medium was collected and VEGF $_{(165,121)}$-protein content was measured using a DuoSet ELISA Development System for human VEGF by R&D Systems (DY293) on a Perkin Elmer Bio Assay Reader HTS 7000.

RNA Isolation and Reverse Transcription

Monolayers were extracted using 500 μl of TRI-Reagent (MRC, Inc. TR-118) with 5 μl/ml of Polyacryl-carrier (MRC, Inc. PC-152) for 10 min at RT and transferred to Eppendorf tubes. 1-Bromo-3-Choro-Propane (150 ml) was added, the tubes vortexed for 15 s, and then centrifuged at 12,000*g* for 15 min at 4°C. The colorless layer on the top was transferred into a new tube and 750 μl of isopropanol was added, then the tube was centrifuged at 12,000*g* for 10 min at 4°C. Sample supernatants were removed and pellets were washed in 1 ml of 75% EtOH by vortexing and centrifuging at 10,000*g* for 5 min at 4°C. Pellets were air-dried and re-suspended in 30 μl of DEPC-treated water. After 15 min at 60°C, tubes were immediately placed on ice. The total RNA amount and purity were assessed by measuring the absorbance at 260 nm and 280 nm. Reverse transcription was performed using 1 μg of total RNA sample, which was mixed with 2 μl of 10× TaqMan RT Buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 4.4 μl of 25 mM magnesium chloride, 4 μl of dNTP mixture (2.5 mM of each dNTP), 1 μl of random hexamers (50 μM), 0.4 μl of RNase inhibitor (20 U/ml), and 0.5 μl of MultiScribe Reverse Transcriptase (50 U/μl) (all from Applied Biosystems, Foster City, CA); DEPC-treated water was added to bring the final reaction volume to 20 μl. Reverse transcription was performed using a Thermal Cycler 9600 by Applied Biosystems. cDNA samples were appropriately diluted with Tris-EDTA buffer before being used for real-time RT PCR.

Real-Time RT PCR

Oligonucleotide primers and TaqMan probes were designed using the Primer Express Oligo Design software (Ver. 1.5, Applied Biosystems). The nucleotide sequences were obtained from the GenBank database and the probes were designed to overlap an exon–exon junction in order to avoid amplification of genomic DNA (Table I). Primers and probes for amplification of 18S ribosomal RNA, used as endogenous control, were from Applied Biosystems. All other primers and labeled TaqMan probes were from Microsynth (Balgach, CH). TaqMan probes were labeled with the reporter dye molecule FAM (6-carboxyfluorescein) at the 5′end and with the quencher dye TAMRA (6-carboxy-N, N, N′, N′-tetramethylrhodamine) at the 3′end. The PCR reaction mixture contained TaqMan Universal PCR master mix without AmpErase UNG (Applied Biosystems), 900 nM primers (forward and reverse), 250 nM TaqMan probe, and 2 μl of cDNA sample for a total reaction volume of 25 μl. PCR conditions were 95°C for 10 min, followed by 42 cycles of amplification at 95°C for 15 s and 60°C for 1 min using the GeneAmp 5700 Sequence Detection System

TABLE I. Primers and Probes for Real-Time RT-PCR

Target gene	Sequence (5′ → 3′)
Collagen I	
Forw	CCC TGG AAA GAA TGG AGA TGA T
Rev	ACT GAA ACC TCT GTG TCC CTT CA
Probe	CGG GCA ATC CTC GAG CAC CCT
Osteonectin	
Forw	ATC TTC CCT GTA CAC TGG CAG TTC
Rev	CTC GGT GTG GGA GAG GTA CC
Probe	CAG CTG GAC CAG CAC CCC ATT GAC
MMP-13	
Forw	CGG CCA CTC CTT AGG TCT TG
Rev	TTT TGC CGG TGT AGG TGT AGA TAG
Probe	CTC CAA GGA CCC TGG AGC ACT CAT GT
BMP-2	
Forw	AAC ACT GTG CGC AGC TTC C
Rev	CTC CGG GTT GTT TTC CCA C
Probe	CCA TGA AGA ATC TTT GGA AGA ACT ACC AGA AAC TG
Osteocalcin	
Forw	AAG AGA CCC AGG CGC TAC CT
Rev	AAC TCG TCA CAG TCC GGA TTG
Probe	ATG GCT GGG AGC CCC AGT CCC
Osteopontin	
Forw	CTC AGG CCA GTT GCA GCC
Rev	CAA AAG CAA ATC ACT GCA ATT CTC
Probe	AAA CGC CGA CCA AGG AAA ACT CAC TAC C
Runx2	
Forw	AGC AAG GTT CAA CGA TCT GAG AT
Rev	TTT GTG AAG ACG GTT ATG GTC AA
Probe	TGA AAC TCT TGC CTC GTC CAC TCC G
BSP II	
Forw	TGC CTT GAG CCT GCT TCC
Rev	GCA AAA TTA AAG CAG TCT TCA TTT TG
Probe	CTC CAG GAC TGC CAG AGG AAG CAA TCA

OSX, SOX 9, collagen II, PPARγ, and GR (both isoforms α and β). Primers and Probes: Assay-On-Demand by Applied Biosystems (Assays: Hs00541729_m1, Hs00165814_m1, Hs00264051_m1, Hs00234592_m1, Hs00230813_m1). Probes were modified at the 5′ end with the FAM fluorescent dye (6-carboxyfluorescein) and at the 3′ end with the TAMRA fluorescent dye (6-carboxy-N,N,N′,N′-tetramethylrhodamine).

(Applied Biosystems). Relative quantification of mRNA targets was performed according to the comparative C_T method with 18S ribosomal RNA as endogenous control (ABI PRISM 7700 Sequence Detector User Bulletin [2], PE Applied Biosystems 1997).

Van Kossa Staining

BMSC monolayers were rinsed with TBSS. A silver nitrate solution (5%) was added and the cells were exposed to strong light for 20 min. After rinsing three times with distilled water, the cells were incubated in fresh 5% sodium thiosulfate for 10 min. After rinsing three times with distilled water, 0.1% nuclear fast red solution was added and the cells were incubated for 10 min, before being washed again with distilled water. Samples were left in distilled water and pictures were taken immediately.

Statistical Analysis

The data are expressed as mean ± standard error unless stated otherwise. Statistics were performed using the non-parametric Mann–Whitney U-test, which compares the medians of two independent distributions. $P < 0.05$ was considered statistically significant.

RESULTS

Bone marrow aspirates from seven human donors (age range 44–66 years) were obtained. Each experiment was done with cells from at least three different donors. Due to the high variability in the osteogenic potential of BMSC between donors, data is shown from one representative experiment, although the same trends were observed in all three experiments, and they are summarized in Table II.

The BMSC used for the experiments were expanded using FGF-2 to increase the cell number while keeping them in a tripotential state capable of osteogenic, chondrogenic, and adipogenic differentiation [Martin et al., 1997; Muraglia et al., 2000; Bianchi et al., 2003].

Indirect Contact Co-Culture Versus Conditioned Medium Culture

Two different types of HUVEC-BMSC culture systems were used; the first, using a trans-well

TABLE II. Summary of the Mean Data Observed in Each Single Experiment

Col 1 gene expression	Day 24 rel. to BMSC in OM		
Condition	A	B	C
BMSC in OM	1.00	1.00	1.00
BMSC in OM with HUVEC	0.59	0.59	0.73
BMSC in OM with HUVEC (VEGF)	0.49	0.54	0.52
ON gene expression	Day 24 rel. to day 1 and to BMSC in OM		
Condition	A	B	C
BMSC in OM	1.00	1.00	1.00
BMSC in OM with HUVEC	0.28	0.52	0.69
BMSC in OM with HUVEC (VEGF)	0.25	0.20	0.65
BMP-2 gene expression	Day 24 rel. to day 1 and to BMSC in OM		
Condition	A	B	C
BMSC in OM	1.00	1.00	1.00
BMSC in OM with HUVEC	0.24	0.17	0.78
BMSC in OM with HUVEC (VEGF)	0.16	Not detectable	0.69
MMP-13 gene expression	Day 24 rel. to day 1 and to BMSC in OM		
Condition	A	B	C
BMSC in OM	1.00	1.00	1.00
BMSC in OM with HUVEC	0.55	0.01	0.99
BMSC in OM with HUVEC (VEGF)	0.06	0.01	0.71

system, allowed indirect two-way communication between the two cell types; and the second, HUVEC-conditioned medium, allowed one-way communication from HUVEC to BMSC but not vice versa. Since no significant differences in the results could be observed between the two culture types, we will not distinguish anymore between them, and we will generally discuss our results as: Effect of HUVEC on BMSC Differentiation.

Effect of HUVEC on BMSC Differentiation in Non-OM

In preliminary experiments, the effect of HUVEC on BMSC differentiation when cultured in non-OM was investigated. HUVEC did not show any effect on ALP activity or matrix mineralization of BMSC compared to the control (no HUVEC). This suggested that HUVEC were not able to replace the positive effect of dexamethasone on BMSC differentiation into osteoblasts in these in vitro culture systems (data not shown).

Effect of HUVEC on BMSC Differentiation in OM

As a positive control, BMSC were cultured in OM without HUVEC. As expected, this led to an increase of several osteoblastic markers, such as matrix mineralization and ALP activity (Figs. 1–3). Furthermore, we observed an elevated expression of typical early and late osteoblastic marker genes including collagen 1 (COL1), osteonectin (ON), matrix metalloproteinase 13 (MMP-13), and bone morphogenetic protein 2 (BMP-2), when compared to BMSC cultured in non-OM (Fig. 4a–d).

The addition of non-stimulated HUVEC to BMSC cultures significantly decreased ALP activity (Fig. 1), matrix mineralization (Figs. 2 and 3), and mRNA levels of COL1, ON, MMP-13, and BMP-2 (Fig. 4). There was a slight increase in the DNA content of BMSC upon addition of non-stimulated HUVEC, but this increase was not significant (data not shown).

Osterix was expressed at almost undetectable levels early in culture. After 24 days, BMSC cultured in non-OM still showed low OSX expression, while BMSC in OM expressed OSX prominently. HUVEC significantly decreased OSX gene expression of BMSC, suggesting an inhibitory effect on BMSC differentiation (Fig. 5). Interestingly, Sox-9 expression levels dropped with culture time and reached almost undetectable levels in all cultures supplemented with OM, including those with HUVEC (Fig. 6).

When VEGF-stimulated HUVEC were added to BMSC, we observed an even more inhibitory effect on the measured markers than with non-stimulated HUVEC (Figs. 1–6).

We have also evaluated osteopontin (OP), osteocalcin (OC), bone sialoprotein II (BSP), Runx2, and glucocorticoid receptor (GR) gene expression. OP was highest at the very beginning of all cultures, then dropped and rose again with time in culture. Interestingly, BMSC cultured in non-OM showed higher OP expression than BMSC in OM (data not shown). When BMSC were cultured with HUVEC, OP

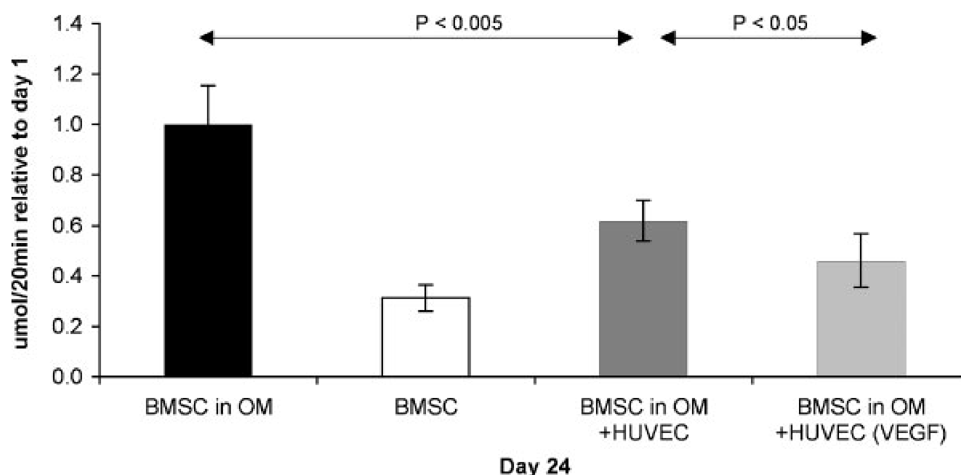

Fig. 1. Representative graph of ALP activity of bone marrow stromal cell (BMSC): BMSC grown in osteogenic medium (OM) showed a clear increase in ALP activity compared to BMSC in non-OM. Human umbilical vein endothelial cells (HUVEC) significantly ($P < 0.005$) reduced the increase in ALP activity of BMSC. Vascular endothelial growth factor (VEGF)-stimulated HUVEC further reduced ALP activity ($P < 0.05$). Statistical analyses were performed for day 24 (n = 6).

expression was further decreased. OC expression was constantly low in all culture systems, and almost undetectable in OM. Addition of HUVEC didn't show any effect on OC (data not shown). BSP gene expression was decreased by HUVEC (data not shown). Runx2 expression was always higher in cultures with OM and slightly increased in all cultures over time. HUVEC didn't change this pattern (data not shown). GR expression by BMSC was evaluated at different time points when cultured in OM and non-OM with or without HUVEC stimulation. No significant differences were observed between the different culture conditions on GR expression levels (data not shown).

Recovery Experiment

In order to evaluate whether the observed inhibitory effect of HUVEC on BMSC differentiation was reversible, we cultured BMSC in

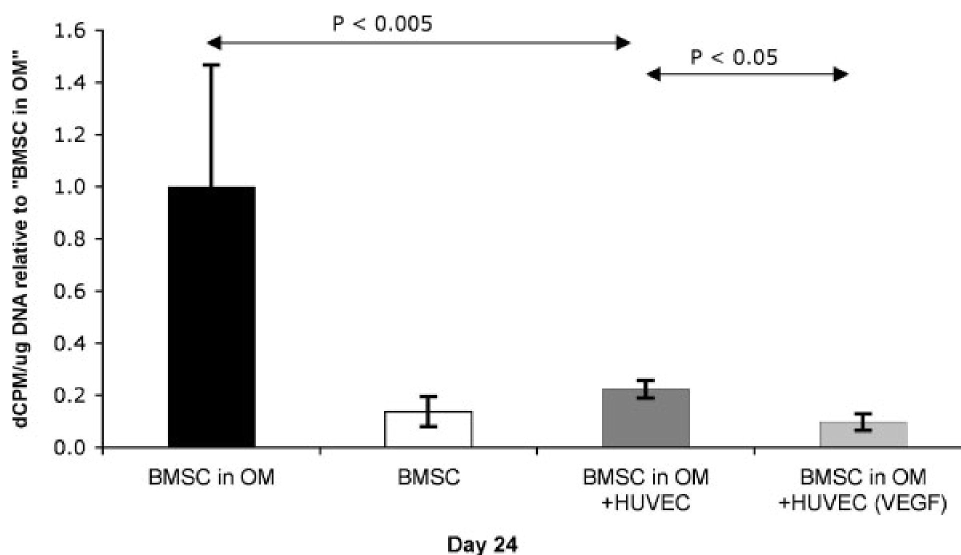

Fig. 2. ^{45}Ca isotope incorporation of BMSC at day 24 of culture (n = 9): BMSC grown in OM showed high matrix mineralization, while BMSC in non-OM did not. Cultures of BMSC and HUVEC showed a significant decrease ($P < 0.005$) in matrix mineralization when compared to BMSC grown alone. This inhibitory effect was enhanced, when HUVEC were previously stimulated with VEGF ($P < 0.05$).

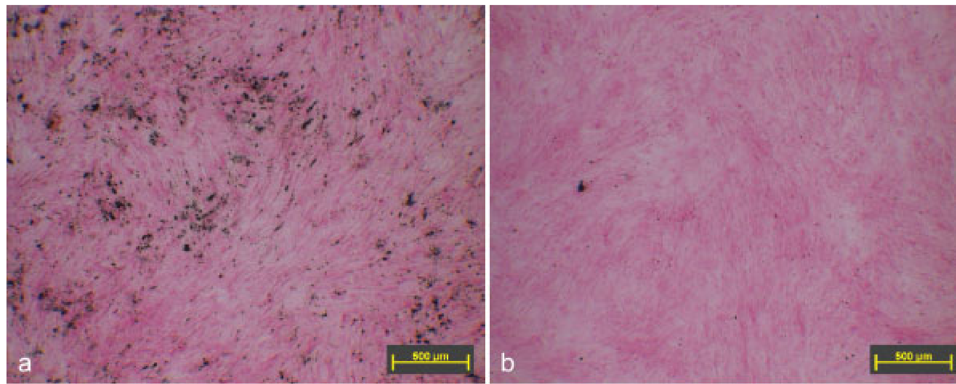

Fig. 3. Van Kossa staining of BMSC grown in OM (**a**) and BMSC grown in OM supplemented with HUVEC-conditioned medium (**b**). Calcium deposition is clearly inhibited by the presence of HUVEC.

OM with the addition of non-stimulated HUVEC-conditioned medium (1:10) and replaced it at different time points with only OM. As expected, BMSC that were exposed to OM containing non-stimulated HUVEC-conditioned medium (1:10) during the whole experiment showed inhibition of $^{45}Ca^{+2}$ incorporation. Upon removal of non-stimulated HUVEC-conditioned medium, all these BMSC cultures showed matrix mineralization similar to the positive control (Fig. 7). Interestingly, BMSC that were in contact with HUVEC-conditioned medium for a longer period of time (17 and 24 days) incorporated more calcium into their matrix upon HUVEC-conditioned medium removal, than BMSC that were in contact with HUVEC-conditioned medium for a shorter period of time (3 and 10 days) (Fig. 7). OSX gene expression was also analyzed and showed a similar picture (data not shown).

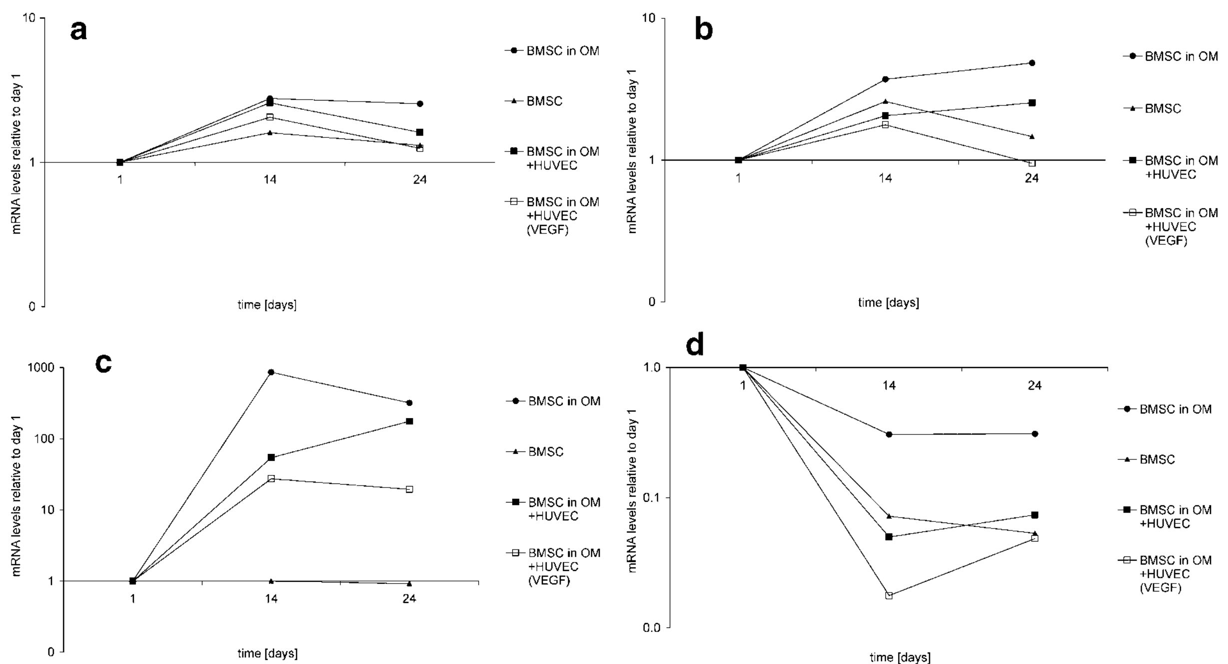

Fig. 4. Representative graphs of mRNA levels of collagen 1 (COL1) (**a**), osteonectin (ON) (**b**), matrix metalloproteinase 13 (MMP-13) (**c**), and bone morphogenetic protein 2 (BMP-2) (**d**) measured by real-time RT-PCR: BMSC cultured in OM resulted in an upregulation of osteoblastic marker genes compared to BMSC in non-OM. Cultures with HUVEC decreased the expression of these marker genes significantly, an effect that was enhanced, when HUVEC were previously stimulated with VEGF. Day 24 data from all three experiments are summarized in Table II.

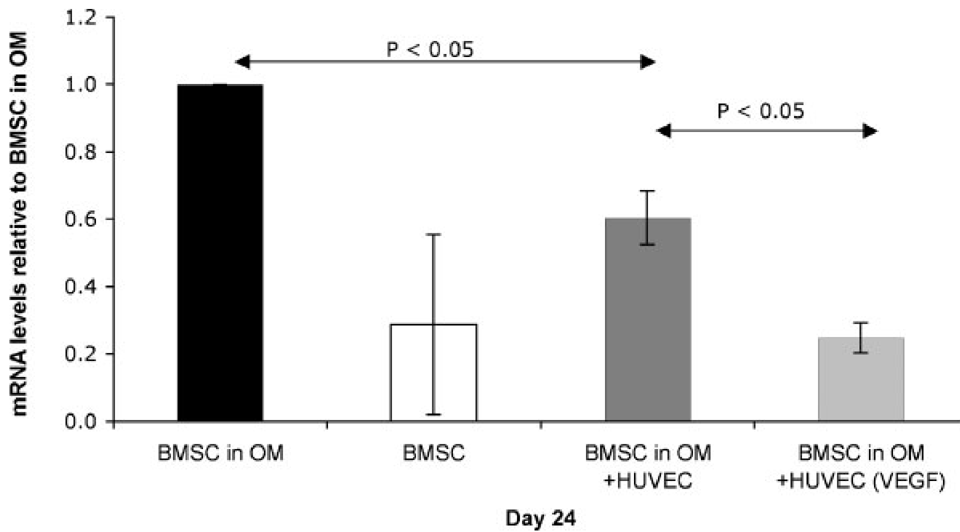

Fig. 5. Osterix (OSX) mRNA levels relative to BMSC in OM at day 24 (n = 4): At day 1, OSX mRNA levels were almost undetectable in all cultures (data not shown). At day 24, BMSC grown in non-OM showed low OSX expression, while BMSC grown in OM showed elevated OSX expression. This expression was significantly downregulated by HUVEC ($P < 0.05$). Again, VEGF-stimulated HUVEC showed a more pronounced inhibitory effect ($P < 0.05$).

Inhibition Experiment

To investigate at which times HUVEC could interfere with the BMSC differentiation process, we cultured BMSC in OM and then replaced it with OM containing non-stimulated HUVEC-conditioned medium (1:10) at different time points. The analysis of OSX gene expression showed, that osteoblastic differentiation was suppressed shortly after the medium was switched to medium containing non-stimulated HUVEC-conditioned medium (1:10). Even after 21 days of osteogenic stimulation, addition of non-stimulated HUVEC-conditioned medium

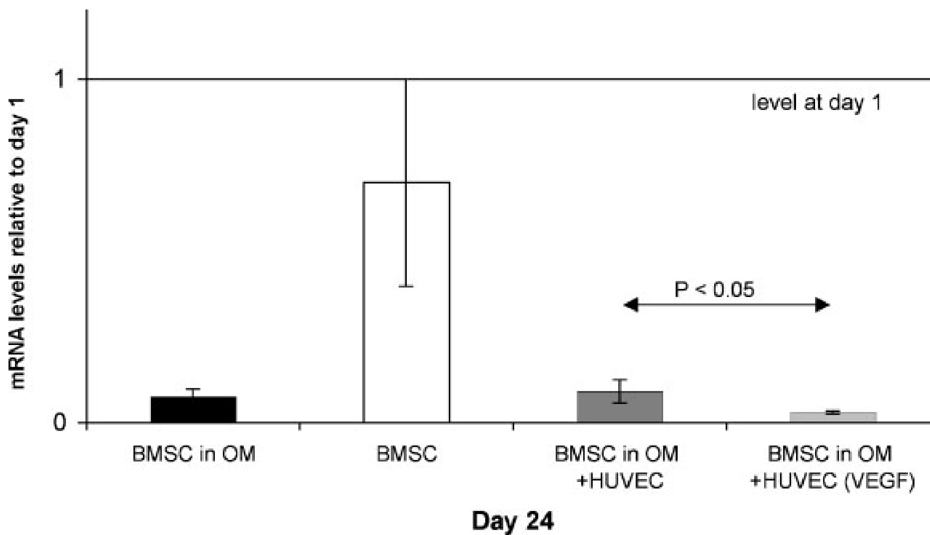

Fig. 6. mRNA levels of the chondrocytic marker Sox-9 at day 24 relative to day 1 (n = 4): The expression of Sox-9 dropped significantly after 24 days in all cultures, suggesting that none of these culture conditions propagates a chondrocytic phenotype. However, BMSC cultured in OM showed a more significant reduction of Sox-9 expression than BMSC cultured in non-OM. Unstimulated HUVEC had no effect on the inhibition of Sox-9 expression by OM, but VEGF-stimulated HUVEC significantly further decreased Sox-9 expression of BMSC ($P < 0.05$). Collagen 2 (COL2), another chondrocytic marker, was almost undetectable in all cultures during the whole experiment (data not shown).

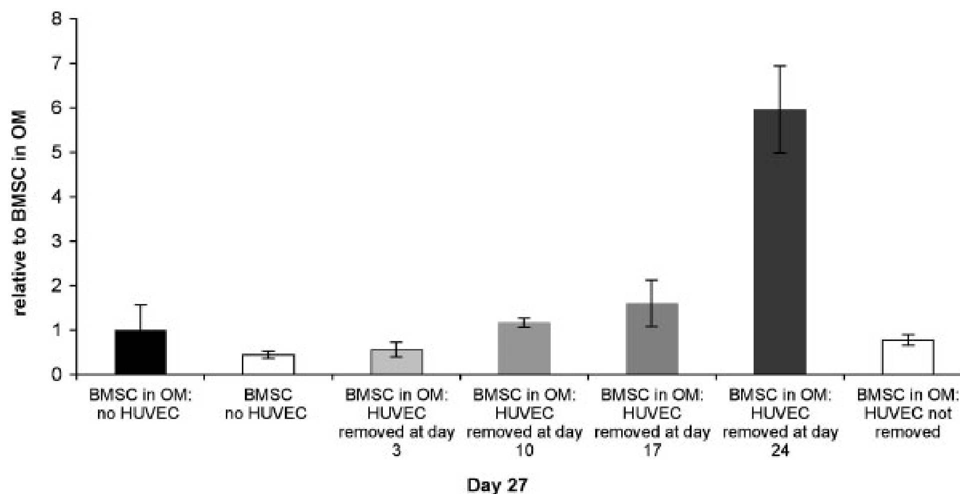

Fig. 7. ^{45}Ca incorporation of BMSC in OM after removal of HUVEC at different time points (n = 4): Calcium deposition by BMSC was inhibited by HUVEC, but upon removal of HUVEC, BMSC showed again significant matrix mineralization. Interestingly, it seemed that the longer BMSC were cultured with HUVEC-conditioned medium, the higher was the amount of ^{45}Ca incorporated into their extracellular matrix, after removal of HUVEC-conditioned medium (a). OSX gene expression showed a similar response upon removal of HUVEC-conditioned medium (data not shown).

resulted in a four to five times decrease in OSX expression at day 28 compared to BMSC in OM (Fig. 8b). ALP activity showed a similar pattern (Fig. 8a).

Chondro-CM and Fibro-CM on BMSC

To investigate, if the inhibitory effect described in the previous sections was specific to HUVEC, we cultured BMSC in OM supplemented with Chondro-CM as well as with Fibro-CM. Both Chondro-CM and Fibro-CM did not alter the calcium incorporation by BMSC or the expression of OSX, while HUVEC-CM, in accordance with the results described above, was able to significantly downregulate calcium incorporation as well as OSX gene expression (Fig. 9a,b).

VEGF Protein Levels in Culture Media

We have measured the VEGF protein levels at several time points in the culture supernatant to investigate whether VEGF concentrations would be altered in the co-cultures compared to BMSC alone. The highest amount of VEGF was observed in BMSC cultured in non-OM, while in OM VEGF was downregulated. HUVEC had no effect on VEGF protein levels measured in the culture supernatant (data not shown).

DISCUSSION

In the present study, we have investigated the effect of HUVEC on BMSC differentiation towards the expression of an osteoblastic phenotype. The differentiation of BMSC by OM was inhibited following exposure to HUVEC. This was reflected by a significant decrease in matrix mineralization, ALP activity, and in reduced expression of specific osteoblastic genes, including MMP-13, COL1, BMP-2, and ON. However, no changes were observed in GR expression, indicating that the inhibition of BMSC differentiation by HUVEC was not mediated via the GR. Interestingly, OC gene expression seems not to be an appropriate differentiation marker in human cells, as previously reported [Frank et al., 2002].

Human umbilical vein endothelial cells were co-cultured with BMSC for up to 28 days in two different types of culture systems: indirect contact and HUVEC-conditioned medium. The direct contact co-culture system was not used due to the difficulty of identifying specific markers of either HUVEC or BMSC when cultured together, as well as due to the lack of a suitable culture medium that could support the growth of BMSC and HUVEC together for more than just a few days, without addition of growth factors (EGF, PDGF, VEGF, bFGF ...). Interestingly, the observed inhibitory effect of HUVEC on BMSC differentiation was seen in both co-culture systems, indicating that HUVEC secrete one or several factors that can delay BMSC differentiation. Furthermore, it appears that this factor(s) acts by inhibiting OSX expression and therefore arresting BMSC

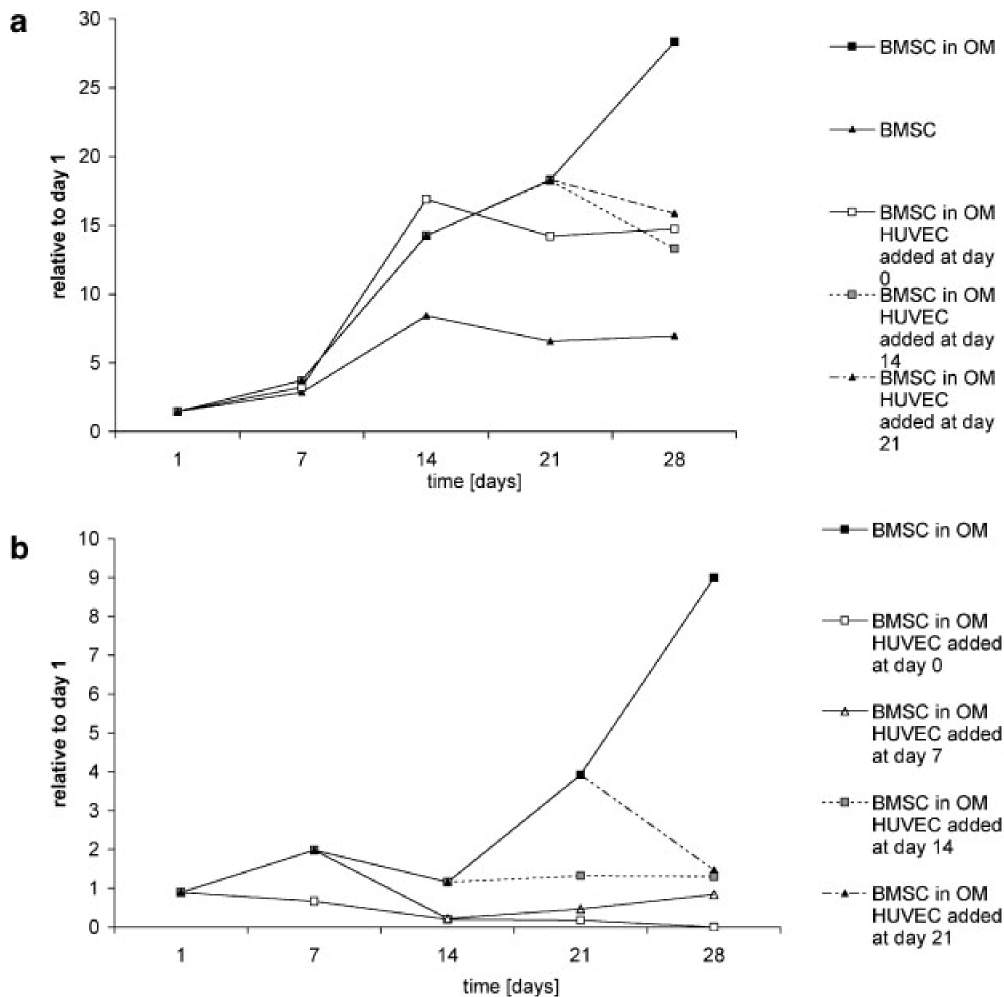

Fig. 8. Representative graphs of ALP activity (**a**) and OSX gene expression (**b**) of BMSC: At different time points, the OM was replaced by HUVEC-conditioned medium. The differentiation process was suppressed by the addition of HUVEC-conditioned medium, as judged by the inhibition of ALP activity (a) and suppression of OSX expression levels (b).

differentiation at a pre-osteoblastic stage (Fig. 10). It has been shown that Runx2 plays a role in the commitment-step to osteo-chondro progenitor cells whereas OSX acts mainly on the terminal differentiation of osteoblasts and on distinguishing the osteogenic pathway from the chondrogenic one [Nakashima et al., 2002]. This might explain why Runx2 is not affected by HUVEC. The suppression of OSX expression by HUVEC could even be induced at later stages of BMSC differentiation, just before mineralization of the extracellular matrix would have started. In addition, the reversibility of the HUVEC inhibitory effect on BMSC differentiation was demonstrated by our recovery experiment, which showed that upon removal of HUVEC-conditioned medium, BMSC could rapidly further differentiate towards the expression of an osteoblastic phenotype. Interestingly, this last experiment also indicates that BMSC exposed to HUVEC for longer periods of time, seem to differentiate even faster towards an osteoblastic phenotype upon removal of HUVEC, when compared to BMSC that have never been in contact with HUVEC (Fig. 7). It is also important to note that BMSC exposed to HUVEC-CM showed no COL2 expression (data not shown) and extremely low expression of Sox-9, two typical markers of the chondrogenic phenotype (Fig. 6). There was also no increase in the expression of the adipocytic marker PPAR-gamma over time (data not shown). These observations suggest that HUVEC-conditioned medium does not drive BMSC towards the expression of a chondrogenic or an adipogenic phenotype. Together, our data from this

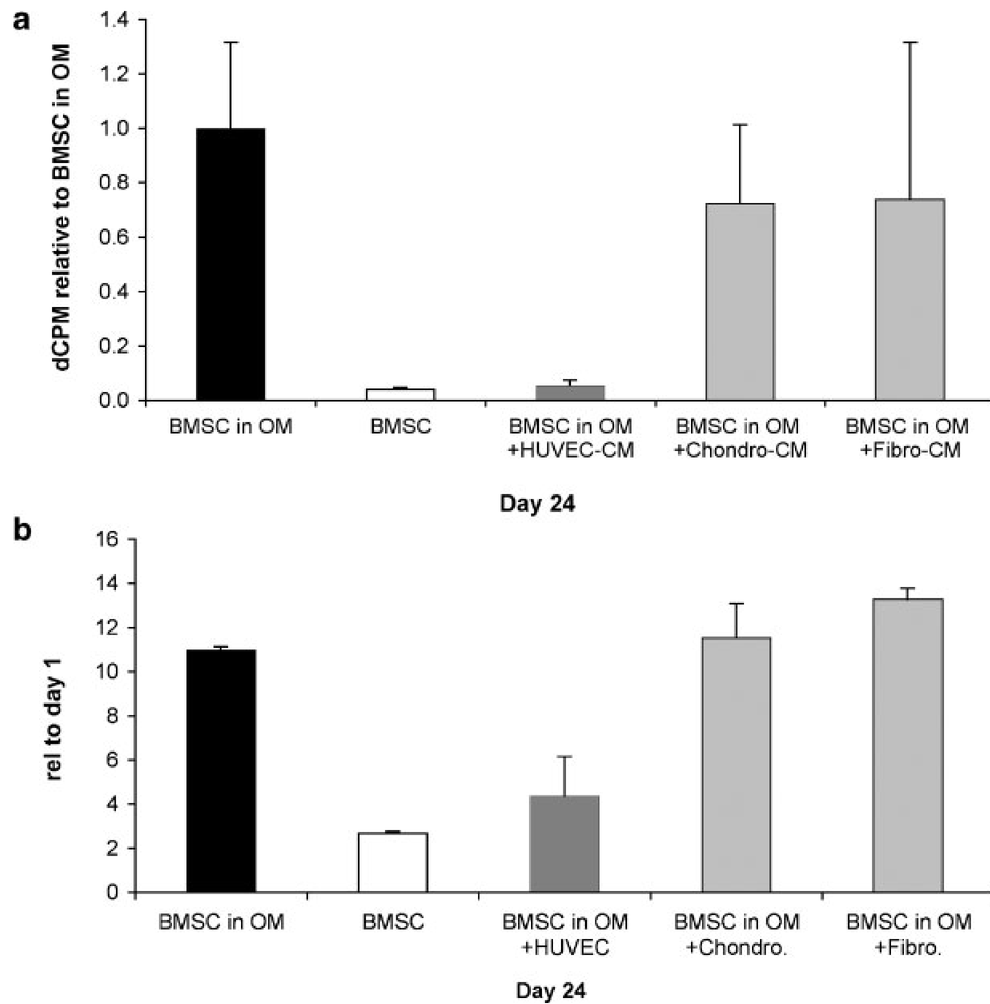

Fig. 9. ^{45}Ca incorporation (**a**) and OSX gene expression (**b**) of BMSC in OM supplemented with endothelial- (HUVEC-CM), chondrocyte- (Chondro-CM) or fibroblast-conditioned medium (Fibro-CM) (a: n = 3, b: n = 3). ^{45}Ca incorporation was significantly downregulated by HUVEC-CM ($P < 0.05$), while Chondro-CM or Fibro-CM had no effect. OSX gene expression showed the same pattern ($P < 0.05$).

in vitro system suggest that endothelial cells can increase the osteogenic potential of osteoprecursor cells while keeping them in a pre-osteoblast state (but at a later stage than the bipotential precursor stage for chondrocytes and osteoblasts, since Runx2 expression is unchanged by HUVEC). In addition, this effect is specific to HUVEC, since chondro-CM as well as Fibro-CM did not show this inhibitory effect on BMSC. This would correlate with the in vivo potential of endothelial cells to recruit large numbers of osteogenic precursor cells at sites of bone modeling or remodeling. Further studies are necessary to verify this speculation.

Few studies have focused on the interactions between endothelial cells and osteoblastic cells in the past years, and the outcomes of these studies were somewhat divergent. While the positive influence of endothelial cells on osteoblastic cell proliferation has been reported quite consistently, their influence on osteoblastic cell differentiation seems rather controversial [Jones et al., 1995; Wang et al., 1997; Villars et al., 2000]. Wang et al. [1997] reported an increase in ALP activity in human osteoblast-like cells when cultured in HUVEC-conditioned medium, while Jones et al. [1995] showed a decrease in ALP activity of BMSC when cultured on EC feeder layers. Villars et al. [2000] showed a decrease in ALP activity in all non-direct contact co-cultures, but an increase in ALP activity in direct contact co-cultures, but also a decrease in OC synthesis in direct contact co-cultures. Kaigler et al. [2005] showed

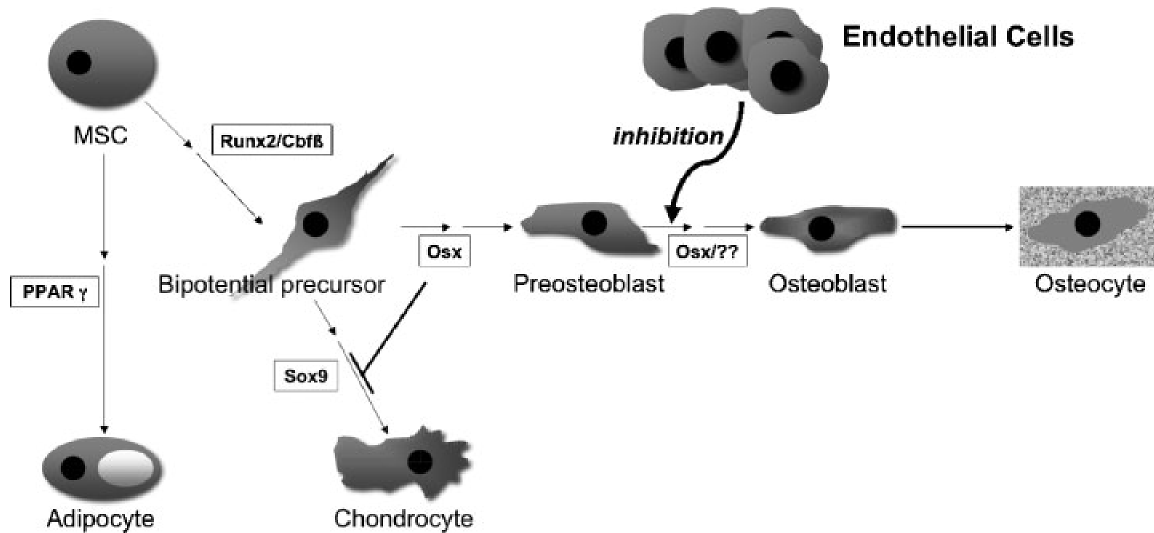

Fig. 10. A simplified schematic representation of the different stages of the differentiation of MSC into osteoblasts: We postulate that HUVEC may have the ability to arrest osteoblastic differentiation in a pre-osteoblastic stage by inhibiting OSX gene expression. The inhibitory effect of HUVEC does not result in an increase of chondrocytic or adipocytic markers. In vivo, this could lead to an accumulation of pre-osteoblasts at sites of bone turnover and repair.

increased ALP activity as well as OC secretion only when BMSC and EC were co-cultured in direct contact. Guillotin et al. [2004] showed that different kinds of primary endothelial cells were able to induce ALP activity of osteoprogenitors when co-cultured in direct contact for up to 6 days, while transformed endothelial cells lines showed no effect. Stahl et al. used EC and osteoblasts in an in vitro 3D spheroidal co-culture model. They described spontaneous organization of osteoblasts and endothelial cells into a core and a surface layer as well as changes in the gene expression patterns of both cell types, including an increase in ALP activity and downregulation of VEGF after 48 h, but did not distinguish between the two cell types during analysis [Stahl et al., 2004; Wenger et al., 2004]. Furthermore, all the studies mentioned above were carried out for a rather short period of time. Our preliminary studies showed no effect of EC on BMSC differentiation in non-OM (in no-direct contact co-cultures), which is in accordance with the above reports.

During the formation of a vascular network, endothelial progenitors originating from hemangioblasts differentiate into mature endothelial cells, which then participate in the processes of vasculogenesis and angiogenesis to form a mature vascular network. Especially in bone, this capillary network is constantly remodeled, which includes destabilization of vessels and sprouting of new branches, processes that are believed to require a change in the differentiation/maturation state of the vessel-covering endothelial cells. Therefore, this change in "maturation" of endothelial cells might influence their effect on BMSC differentiation. These vessel remodeling processes are controlled by several factors, the most important one probably being VEGF [Gale and Yancopoulos, 1999]. Indeed, as observed in the present study, stimulation of HUVEC by VEGF further increases their inhibitory action on BMSC differentiation. This inhibition could not be reproduced by VEGF itself. In fact, we could not detect any VEGF protein (results not shown) in the HUVEC-conditioned media (both VEGF-stimulated and non-stimulated) added to the BMSC, as previously also reported by Villars et al. [2000]. This conclusion is also supported by the observations that VEGF expression in BMSC is decreased by glucocorticoids both in vivo and in vitro; that VEGF protein was detected in BMSC cultures but its level did not show any changes upon addition of HUVEC-conditioned medium or HUVEC in indirect contact; and that Stahl et al. even measured a downregulation of VEGF in their 3D direct contact co-culture model [Stahl et al., 2004; Wenger et al., 2004]. Furthermore, BMSC cultured in non-osteogenic media (no dexamethasone) showed higher levels of VEGF, again indicating that glucocorticoids suppress VEGF expression.

In conclusion, we have shown that HUVEC specifically have an inhibitory effect on dexamethasone-induced BMSC differentiation in vitro. This effect is most probably due to a yet unknown HUVEC secreted factor(s), which suppresses OSX expression, therefore arresting BMSC differentiation at a pre-osteoblastic stage. Furthermore, prior stimulation of HUVEC with VEGF resulted in a further increased inhibitory effect of HUVEC on BMSC differentiation. A tempting speculation arising from these data is that HUVEC might regulate the rate at which BMSC differentiate into osteoblasts, by initiating the recruitment of osteoprecursor cells at sites of bone remodeling and by keeping them in a pre-osteoblastic stage. This would avoid mineral deposition within vessels, but once these pre-osteoblastic cells extravasate out of the vessel and continue their homing to the remodeling/repair site, they will rapidly differentiate into mature osteoblasts and lay down new osteoid tissue.

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