# Co-culture of canine mesenchymal stem cells with primary bone-derived osteoblasts promotes osteogenic differentiation

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# Co-culture of canine mesenchymal stem cells with primary bone-derived osteoblasts promotes osteogenic differentiation

**C. Csaki · U. Matis · A. Mobasheri · M. Shakibaei**

**Abstract** Tissue engineering of bone grafts with osteogenic progenitor cells such as adult mesenchymal stem cells (MSC) represents a promising strategy for the treatment of large bone defects. The aim of this experimental study was to evaluate the osteogenic potential of primary osteoblasts on MSCs in co-culture at different ratios. The co-cultures were treated with or without a specific osteogenic induction medium in monolayer and high density cultures. In monolayer co-cultures, MSCs and osteoblasts actively searched for cell–cell contact leading to cell proliferation and only in treated monolayer co-cultures osteogenesis was observed. Ultrastructural evaluation of high density co-cultures using electron microscopy demonstrated osteogenesis with no significant difference between treated or untreated co-cultures. Immunoblotting confirmed expression of collagen type I, $\beta$1-Integrin, the osteogenic-specific transcription factor Cbfa-1 and induction of the MAPKinase pathway (Shc, Erk1/2) in both treated and untreated co-cultures. Although treatment with the induction medium enhanced osteogenesis in the co-cultures compared to untreated co-cultures, the quality of osteogenesis was proportional to the quantity of osteoblasts in the co-cultures. Fifty percent osteoblasts in the co-cultures markedly increased osteogenesis; even the presence of ten percent osteoblasts in the co-culture strongly promoted osteogenesis. This data leads us to conclude that co-culture of MSCs with osteoblasts combined with the three-dimensional environment of the high density culture strongly promotes osteogenesis and stabilizes the osteogenic potential of MSCs. This approach may prove to be of practical benefit in future tissue engineering and regenerative medicine research.

**Keywords** Co-culture · Mesenchymal stem cell (MSC) · Osteoblast · Osteogenesis · Ultrastructure · Canine

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## Introduction

Large bone defects that occur through trauma, loss of large bone areas after cancer surgery or bone loss through metabolic and degenerative diseases connected with weakening of the whole bone structure and the intrinsic regeneration mechanism, have a limited capacity for self repair. Therefore, such bone defects could benefit from the development of novel treatments that take advantage of readily available and implantable bone grafts (Kajiwara et al. 2005; Beris et al. 2005). Surgical methods such as autologous and/or allogenic osteochondral grafts have been established in surgical medicine and have been used for several decades to fill osteochondrotic defects. Patients with large defects, such as large bone defects after tumour resection, osteonecrosis, extensive trauma or broad focal osteoarthritis (OA) have especially benefited from these grafts (Meyers et al. 1983; Czitrom et al. 1986; Marco et al. 1993). However, the risk of donor site morbidity, infections from allografts

or the sheer size of the implant are important limitations in reconstructive medicine and necessitate development of novel and innovative approaches to overcome these problems (Ross et al. 2000; Silber et al. 2003). A study by Silber et al. evaluating long-term outcome of iliac crest bone graft (ICBG) site morbidity revealed that a large percentage of patients reported chronic donor site pain after anterior ICBG donation (Silber et al. 2003). These limitations have resulted in the development of new strategies to provide bone substitutes from in vitro production through tissue engineered bone constructs. Most of these evolve around bio-compatible and bio-degradable scaffold construction, seeding of scaffolds in vitro with adequate cells such as primary osteoblasts or mesenchymal stem cells (MSCs) and various combinations of growth factors (Krampera et al. 2006). Interestingly, osteoblasts derived from trabecular bone have been shown to posses multilineage differentiation potential, in addition to their osteogenic potential, as long as they are grown in the right culture conditions (Sottile et al. 2002; Song et al. 2005; Noth et al. 2002). MSCs are already on trial for the treatment of aseptic non-traumatic osteonecrosis of the femoral head. Bone marrow implantation into the osteonecrotic zone avoided the progression of the disease and reduced the need for total hip replacement (Gangji et al. 2005; Gangji and Hauzeur, 2005).

MSCs are multipotent cells that not only exhibit chondrogenic and adipogenic potential but also high osteogenic capacities in vitro (Csaki et al. 2007; Jaiswal et al. 1997; Pittenger et al. 1999). They are easily obtained from bone marrow or adipose tissue and they possess osteo-progenitor capacities which means that they can be expanded in vitro for extended periods of time without loosing their differentiation potential (Qu et al. 2007; Yamamoto et al. 2007; Zuk et al. 2001). Indeed, the osteogenic potential in bone marrow derived MSCs seems to be more pronounced compared to the adipogenic or chondrogenic potential. This would suggest that the non-homogenous nature of MSC populations derived from bone marrow could provide a suitable source of cells for bone tissue engineering. Furthermore, the use of adult MSCs provides an alternative and ethically acceptable source of multipotent cells instead of using embryonic stem cells which have proved to be less practical and more controversial.

Several studies have demonstrated that re-implanted MSCs in vivo undergo site specific differentiation and are capable of bone formation. This suggests that tissue specific cells may promote and enhance MSCs to differentiate towards their fully differentiated cell type by promoting dynamic and intensive cell–cell interactions. It further suggests that the differentiation of MSCs is not only modulated by cytokines and hormones but also direct cell-to-cell contacts are vitally important for tissue formation. However, very little is known about the phenotype inducing potential of fully differentiated primary cells such as osteoblasts on multipotent MSCs. It is well known that the MAPKinase pathway plays a pivotal role in osteogenesis in vivo (Jaiswal et al. 2000) and is of vital importance for the maintenance of the cell phenotype (Shakibaei et al. 2001). Furthermore, growth factors such as TGF-$\beta$1, BMP-2 and BMP-4, FGF as well as dexamethasone (Katagiri et al. 1990; Ahrens et al. 1993; Kadiyala et al. 1997; Ducy et al. 2000; McPherron et al. 1999; Mikic et al. 1995), adhesion molecules (Shakibaei 1998; Shakibaei et al. 1999) and the transcription factor Cfba-1 (Ducy 2000) play an important role in osteogenesis. Accordingly, the aim of this study was to evaluate the osteogenic induction potential of primary osteoblasts on autologous MSCs in a co-culture system to gain more insight and a better understanding of the processes involved in osteogenic differentiation of MSCs. This knowledge may help refine and improve tissue engineering of bone constructs with MSCs and may prove to be of practical benefit for research in regenerative medicine.

## Materials and methods

### Antibodies

Polyclonal anti-collagen type I antibody (PAB749P), monoclonal anti-$\beta$1-integrin antibody (MAB1965), polyclonal anti-osteocalcin antibody (PAB1857) and alkaline phosphatase linked sheep anti-mouse (AP303A) and sheep anti-rabbit secondary antibodies (AP304A) for immunoblotting were purchased from Chemicon International, Inc., (Temecula, CA, USA). Polyclonal anti-Cbfa-1 (CBFA11-A) was purchased from Alpha Diagnostics Int. San Antonio, TX, USA. Monoclonal anti-$\beta$-Actin (A4700) was purchased from Sigma, St. Louis, MO, USA). Monoclonal anti-phospho-p42/p44 Erk1/2 antibody (610032) and polyclonal anti-Shc antibody (610082) were purchased from BD (BD Biosciences, Belgium). Gold particle conjugated secondary antibodies were purchased from Amersham (Braunschweig, Germany).

### Isolation and culture of mesenchymal stem cells from bone marrow

Bone marrow mesenchymal stem cells (MSCs) were isolated with density gradient centrifugation using Ficoll-paque (Amersham Bioscience, No. 17-1440-02) from bone marrow obtained during total hip replacement surgeries from the femoral diaphysis of three canines as previously described (Csaki et al. 2007). Briefly, samples were diluted with 5 ml 3% citric acid, further diluted 1:1 with PBS and centrifuged over 15 ml of Ficoll-paque at 400 g/20°C for 30 min. The supernatant was discarded and the middle

layer was washed three times in complete cell culture medium [DMEM/Hams-F12 1:1, 10% FCS, 1% partricin solution, 1% penicillin/streptomycin solution (10,000 IU/10,000 IU), 75 µg/ml ascorbic acid, 1% essential amino acids and 1% Glutamine, all obtained from Seromed (Munich, FRG)]. Cells were plated with 12 ml cell culture medium in a T75 flask and incubated at 37°C/5% $CO_2$. The first medium change was performed after 4 days. Following medium changes were performed three times per week. The flasks were passaged for the first time after five or more confluent colonies had formed. Overall MSCs were passaged up to a maximum of six times.

### Primary osteoblast isolation and culture

Autologous bone fragments were cut from femoral heads obtained during total hip replacement surgeries. Great care was taken to isolate bone fragments only from the inner trabecular bone to avoid contamination with soft or other fibrous tissues. Five to ten ca. 3–4 mm large bone fragments were plated per 35 mm petri dish in 10 ml cell culture medium and incubated at 37°C/5% $CO_2$. After 1 week osteoblasts migrated from the bone fragments and adhered to the cell culture dish. After colonies reached confluency cells were trypsinised. Primary osteoblasts were passaged a total of three times. Medium changes were performed three times per week.

### Monolayer co-cultures

Monolayer cultures and co-cultures were performed on glass cover slips. For monolayer co-cultures MSCs and primary osteoblasts were mixed at a ratio of 9:1, 7:3 and 1:1. Pure cultures of MSCs and primary osteoblast served as controls. Approximately 2,500 cells were plated per glass cover slip and cultures were evaluated after 21 days. Cells were cultured with cell culture medium and with osteogenic induction medium. The osteogenic induction medium was prepared as described (Pittenger et al. 1999); this consisted of DMEM as a base medium, 10% FCS, penicillin/streptomycin solution (10,000 IU/10,000 IU/100 ml), $10^{-7}$ M dexamethasone (Sigma, Cat. No. D-8893), 10 mM $\beta$-Glycerophosphate (Sigma, Cat. No. G-9891) and 50 µM ascorbate-2-phosphate (Sigma, Cat. No. A-8960).

### High density co-cultures

High density cultures and co-cultures were performed as described previously (Shakibaei et al. 1993) with three individual donors. Briefly, high density cultures were established by adding the cells onto a nitrocellulose filter which was placed on a steel-net-bridge. Approximately 8 µl of cell suspension was pipetted onto the nitrocellulose filter and after 1 day in culture this aggregated into a cell pellet. High density cultures and co-cultures were incubated with cell culture medium or with osteogenic induction medium and cells were nurtured through diffusion at the filter medium interface and evaluated after 21 days. In an additional experiment, MSCs were brought into high density cultures and incubated with the medium supernatant obtained from monolayer primary osteoblast cultures. In detail, primary osteoblast cultures were incubated with whole cell culture medium containing 10% FCS overnight. MSCs were then incubated with this medium supernatant. Medium changes were performed every 2 days. MSC high density cultures were evaluated after 14 days with electron-microscopy.

### PKH cell membrane labelling

In order to be able to distinguish the MSCs from the primary osteoblasts in co-culture, to observe their distribution in the high density co-cultures and to monitor their interaction in the monolayer co-cultures, cells were labelled with a fluorescent PKH membrane dye (Sigma). Primary osteoblasts were labelled green with PKH67. Membranes of MSCs were labelled red with PKH26. Labelling was performed according to the manufacturer's recommended protocol. Briefly, 1 million cells were washed three times with PBS to remove all FCS and resuspended in 200 µl of the provided diluent C. Approximately 1 µl of dye was resuspended in 200 µl diluent C and immediately mixed with the cells. After incubation for 4 min the reaction was blocked with 400 µl FCS, the cells transferred to a new tube and washed three times in whole cell culture medium. Successful staining was evaluated under an immunofluorescence microscope and images were digitally captured and stored (Leica).

### Histology

Monolayer cultures were evaluated with von Kossa staining for calcium deposition and evaluated under a light microscope (Leica). High density cultures were embedded in Tissue-Tek (Sakura Finetek Europe, Netherlands), cryopreserved at −80°C, cut into 5–7 µm sections using a cryomicrotome (Zeiss, Germany) and mounted on Superfrost plus slides (Menzel-Glaeser, Germany). Cell distribution in PKH labelled co-cultures was evaluated under a fluorescent microscope (Leica). Photomicrographs were digitally captured and stored.

### Transmission electron microscopy (TEM)

In order to asses the formation of bone matrix in the high density co-cultures at the ultrastructural level, TEM was

performed as previously described in detail (Shakibaei et al. 1997). Briefly, high density co-cultures were fixed for 1 hour in Karnowsky fixative then post-fixed in 1% $OsO_4$ solution. After dehydration in an ascending series of alcohol baths, pellets were embedded in Epon (Plano, Marburg, FRG) and ultra-thin cuts were made on a Reichert-Ultracut E. Ultrathin sections were contrasted with a mixture of 2% uranyl acetate/lead citrate. A transmission electron microscope (TEM 10, Zeiss, Jena, Germany) was used to examine the morphology of the cultures and co-cultures.

### Immunoelectron microscopy (IEM; post embedding technique)

Immunoelectron microscopy was performed as described in detail (Shakibaei 1998). Briefly, cultures were fixed for 1 h in 3% freshly prepared formaldehyde (paraformaldehyde plus 0.25% glutaraldehyde), washed three times with 0.1 M PBS, dehydrated in an ascending series of alcohol baths and embedded in LR-white (Plano, Marburg, FRG). Ultrathin sections of the cultures were prepared and treated as follows: (1) BSA 1% at ambient temperature (AT) for 30 min, (2) testicular chondroitinase (5,000 U/ml) for 5 min to unmask antigen epitopes, (3) PBS/BSA 1%/Tween 20 0.5% 2 × 5 min at AT, (4) primary antibodies (diluted 1:50 in PBS/BSA1%) overnight at 4°C, (5) PBS/BSA 1% 2 × 5 min at AT, (6) secondary antibodies conjugated with goat anti-rabbit immunoglobulin with 10 nm gold-particles (1:50 for 30 min) at AT, (7) after rinsing for 2 × 5 min at AT, (8) contrasting was carried out with tannic acid 1% for 20 min at AT, with osmium 1% for 10 min and with uranyl acetate 2% for 30 min. Finally, the sections were rinsed and examined under a transmission electron microscope (Zeiss, Germany).

### Western blot analysis

For western blot analysis proteins were extracted from the high density cultures and co-cultures with lysis buffer (50 mM TRIS/HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% (v/v) aprotinin, 4 µg/ml pepstatin A, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, PMSF) on ice for 30 min as previously described (Shakibaei et al. 1999, 2001). Total protein content was measured with the bicinchinonic acid system (Uptima, France) using bovine serum albumin (BSA) as a standard. Samples were further reduced with 2-mercaptoethanol and total protein concentrations were adjusted. Proteins were separated with SDS-PAGE under reducing conditions using 5, 7 or 10% gels and then blotted onto a nitrocellulose membrane using a transblot apparatus (Bio-Rad, Munich). Nitrocellulose membranes were blocked for 2 h in 5% (w/v) skimmed milk powder in phosphate buffered saline (PBS)/0.1% Tween 20. Membranes were incubated overnight with the first antibody diluted in blocking buffer at 4°C on a shaker. After washing three times in blocking buffer membranes were further incubated with the alkaline phosphatase conjugated secondary antibodies for 2 h at AT. Following further washing in 0.1 M TRIS, pH 9.5, containing 0.05 M $MgCl_2$ and 0.1 M NaCl, specific antigen–antibody complexes were detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (*p*-toluidine salt; Pierce, Rockford, IL) as substrates for alkaline phosphatase. Immunoblots were semi-quantitatively analysed using the computer program "Quantity one" (Bio-Rad, Munich, Germany). The results are expressed as the mean ± SD of a representative experiment performed in triplicate. Data shown are representative of three independent experiments. The means were compared using Student's *t* test assuming equal variances. Statistical significance was at $P < 0.05$.

## Results

### Interaction between MSCs and primary osteoblasts in PKH labelled co-cultures

Interaction of MSCs and primary osteoblasts in monolayer cultures was observed by labelling the cell types with two different coloured fluorescent membrane dyes (PKH). In 50% MSC/50% primary osteoblast monolayer co-cultures intensive cell-cell contact and interaction was observed (Fig. 1a). After just one day in culture MSCs and primary osteoblasts extended long cytoplasmic processes and actively searched for contacts with the other cell type (Fig. 1a; a, b). Active cell–cell communication continued on the second day (Fig. 1a; c, d), with some primary osteoblasts taking on a multipolar morphology/appearance by displaying long cytoplasmic processes (c). Some cells were already in mitosis, indicating active cell metabolism. Around the third day intensive cell-cell interaction continued and enhanced proliferation of MSCs in the vicinity of primary osteoblasts was observed (Fig. 1a; e, f). This trend continued leading to active proliferation of MSCs around the sixth day (Fig. 1a; g, h) and was observed throughout the eight day culture period (Fig. 1a; i, j).

After bringing the cells into high density co-culture, organisation, nodule formation and matrix deposition were observed with Toluidine blue staining (Fig. 1b; a). Combining PKH labelled MSCs and primary osteoblasts in high density culture, revealed even distribution of the two cell types in the co-cultures (Fig. 1b; b, c). No significant differences in cell distribution were observed between the different ratios. MSCs and primary osteoblasts mixed

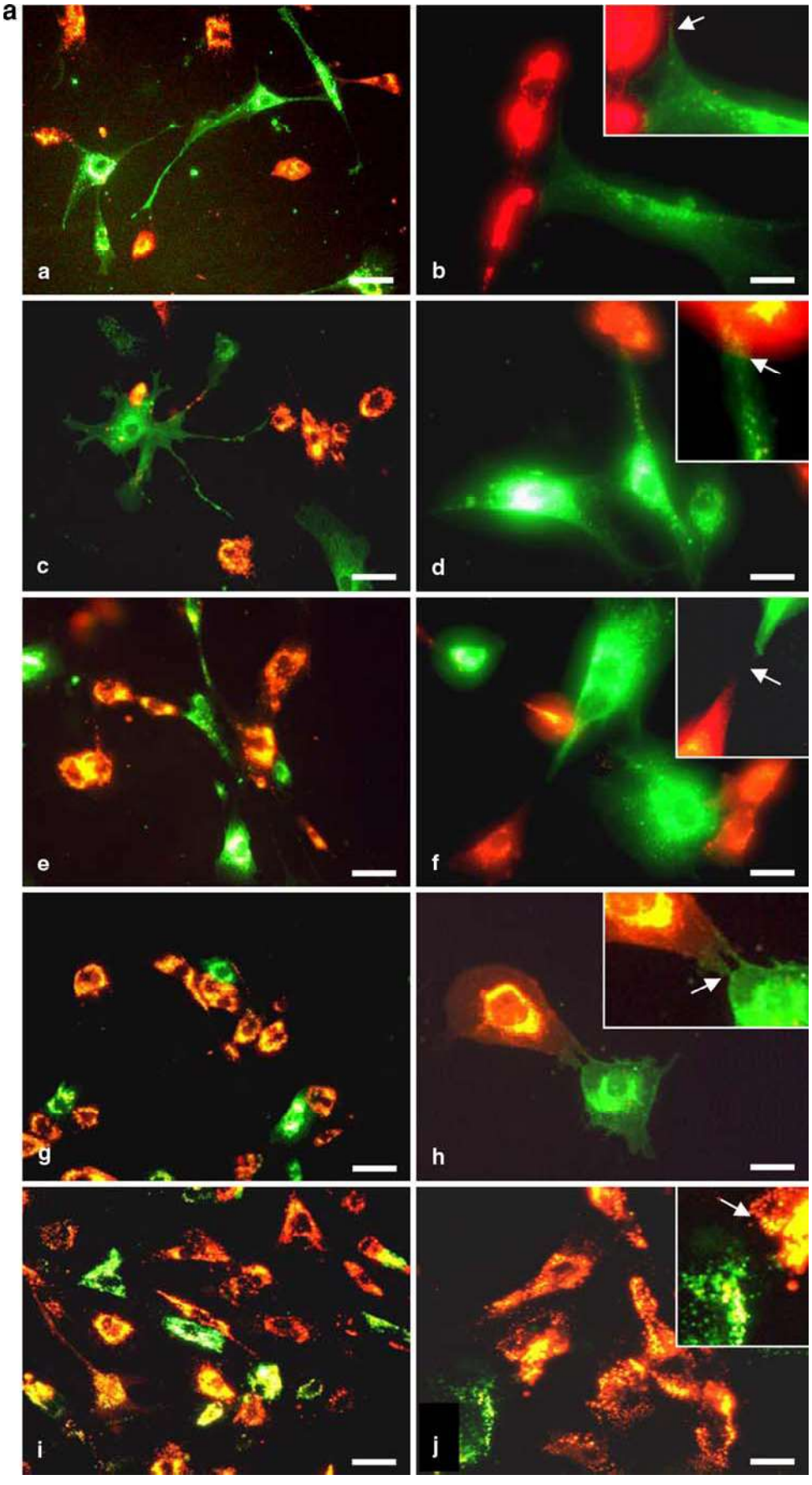
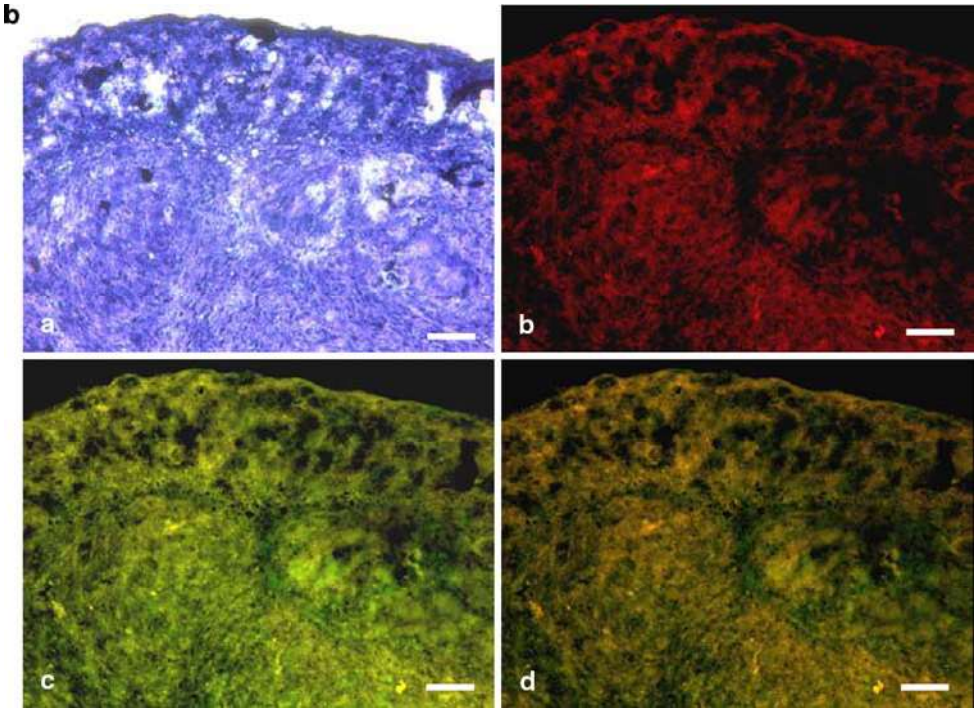



**Fig. 1** Co-culture (50%MSCs/50% primary osteoblasts); fluorescence PKH labelling and Toluidine blue. **a** Monolayer co-cultures. Already after 1 day in co-culture intensive cell-cell contacts and interactions were observed (*a*, *b*), which increased on the second day (*c*, *d*). Strong MSC proliferation was observed around the third day (*e*, *f*), was further enhanced around the sixth day (*g*, *h*) and continued till the end of the culture period at the eighth day (*i*, *j*). **b** High density co-culture. (*a*) Toluidine blue staining demonstrating organisation, nodule formation and matrix deposition in the high density co-culture. (*b*) MSCs are labelled red with PKH-26 and (*c*) primary osteoblasts are labelled green with PKH-67. The immunofluorescence overlaid picture (*d*) of *a* and *b* demonstrates homogenous distribution of both cell types in the high density co-culture. MSCs, *red*; primary osteoblasts, *green*; magnification: **a:** *a*, *c*, *e*, *g*, *i*: ×100, *bar* 60 µm; *b*, *d*, *f*, *h*, *j*: ×200, *bar* 30 µm **b**: *a*, *b*, *c*, *d*: ×400, *bar* 15 µm

**Fig. 1** continued




homogenously and were randomly distributed over the entire area of the high density cultures (Fig. 1; b, d). We did not observe accumulation of MSCs or primary osteoblasts at the borders of the cultures; no clustering of cells was observed in the middle of the cultures.

Osteogenic induction potential in co-culture

*Light microscopy*

Monolayer cultures were stained with von Kossa to evaluate calcium deposition (Fig. 2). In untreated cultures, no calcium deposition was observed in the pure MSCs (Fig. 2; a). In contrast to this, in pure primary osteoblast cultures clustering of the cells and the beginning of nodule formation was observed (Fig. 2; e:arrows). In the untreated co-cultures very weak calcium deposition was observed (Fig. 2; b–d). It can be argued that calcium deposition, although overall very weak, increased slightly from the co-culture with the lowest amount of primary osteoblasts (10% primary osteoblasts/90% MSC) to the co-culture with 50% primary osteoblasts as the ratio of primary osteoblasts increases in the co-cultures.

In contrast to untreated cultures, in the induced monolayer cultures strong von Kossa staining with high amounts of calcium deposition was observed in MSC cultures, primary osteoblast cultures and the various co-cultures (Fig. 2; f–j:arrows). There was no obvious or detectable difference in calcium deposition between induced MSCs, primary osteoblasts or co-cultures.

*TEM*

In order to observe the osteogenic potential of the cells in an environment as close to the in vivo situation as possible, MSCs, primary osteoblasts and the various co-cultures were transferred to the three dimensional environment of high density culture. TEM was used to take high resolution images from the high density cultures after 21 days to evaluate general cell ultrastructure and extracellular matrix formation (Fig. 3).

In untreated high density cultures, MSCs underwent apoptosis, with degeneration of the cells, membrane blebbing, nuclear damage and formation of apoptotic bodies (Fig. 3; a). Treatment of MSCs with induction medium resulted in osteogenesis; cells exhibited high levels of euchromatin in their nuclei, a large number of morphologically normal cellular organelles (mitochondria, rough ER, Golgi apparatus), numerous cell–cell processes and large quantities of thick fibrils of well organised extracellular matrix (Fig. 3; f).

In contrast to untreated MSC cultures, in the other untreated high density cultures, osteogenesis was observed in primary osteoblast cultures as well as in all the co-cultures (Fig. 3; b–e). Further, treatment of primary osteoblast cultures and the various co-cultures with the osteogenic induction medium resulted in osteogenesis (Fig. 3; g–j).

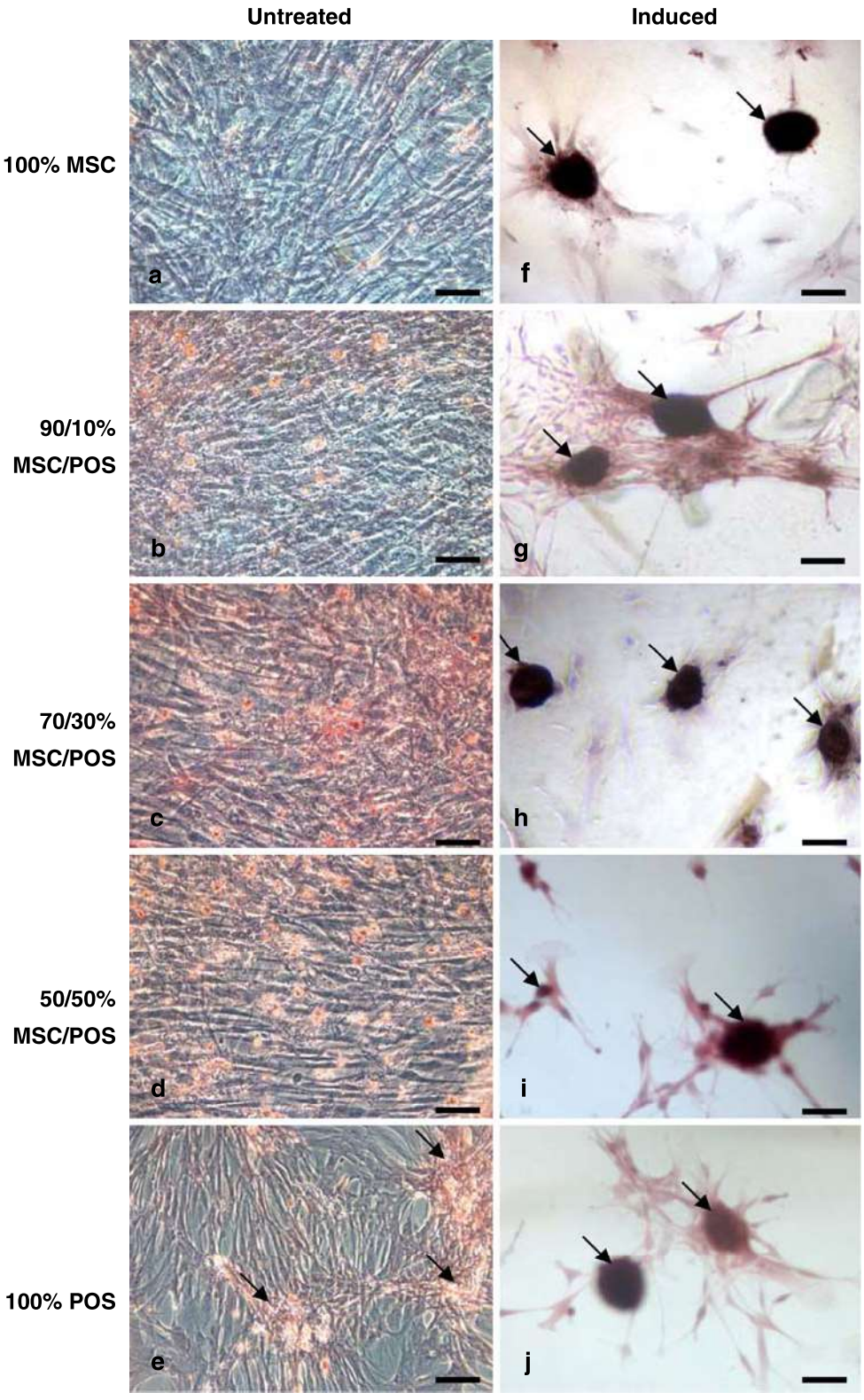



**Fig. 2** Monolayer Co-culture: light microscopy von Kossa staining; calcium deposition was evaluated with von Kossa staining after 21 days in monolayer culture. In untreated primary osteoblast cultures (*e*) only clustering of the cells and the beginning of nodule formation (*arrows*) was observed. Calcium deposition was neither observed in untreated MSCs (*a*) nor in untreated co-cultures (*b*, *d*). In contrast to this, strong calcium deposition (*arrows*) was observed in treated MSC cultures (*f*), primary osteoblast cultures (*j*) and the various co-cultures (*g–i*). There was no significant detectable difference in calcium deposition between induced MSC cultures, primary osteoblast cultures or co-cultures. ×200, *bar* 30 μm

However, no significant differences in osteogenesis were observed on the ultrastructural level between untreated primary osteoblast cultures and the untreated co-cultures. Further, no differences were noted between the untreated co-cultures in comparison to treated primary osteoblasts or treated co-cultures.

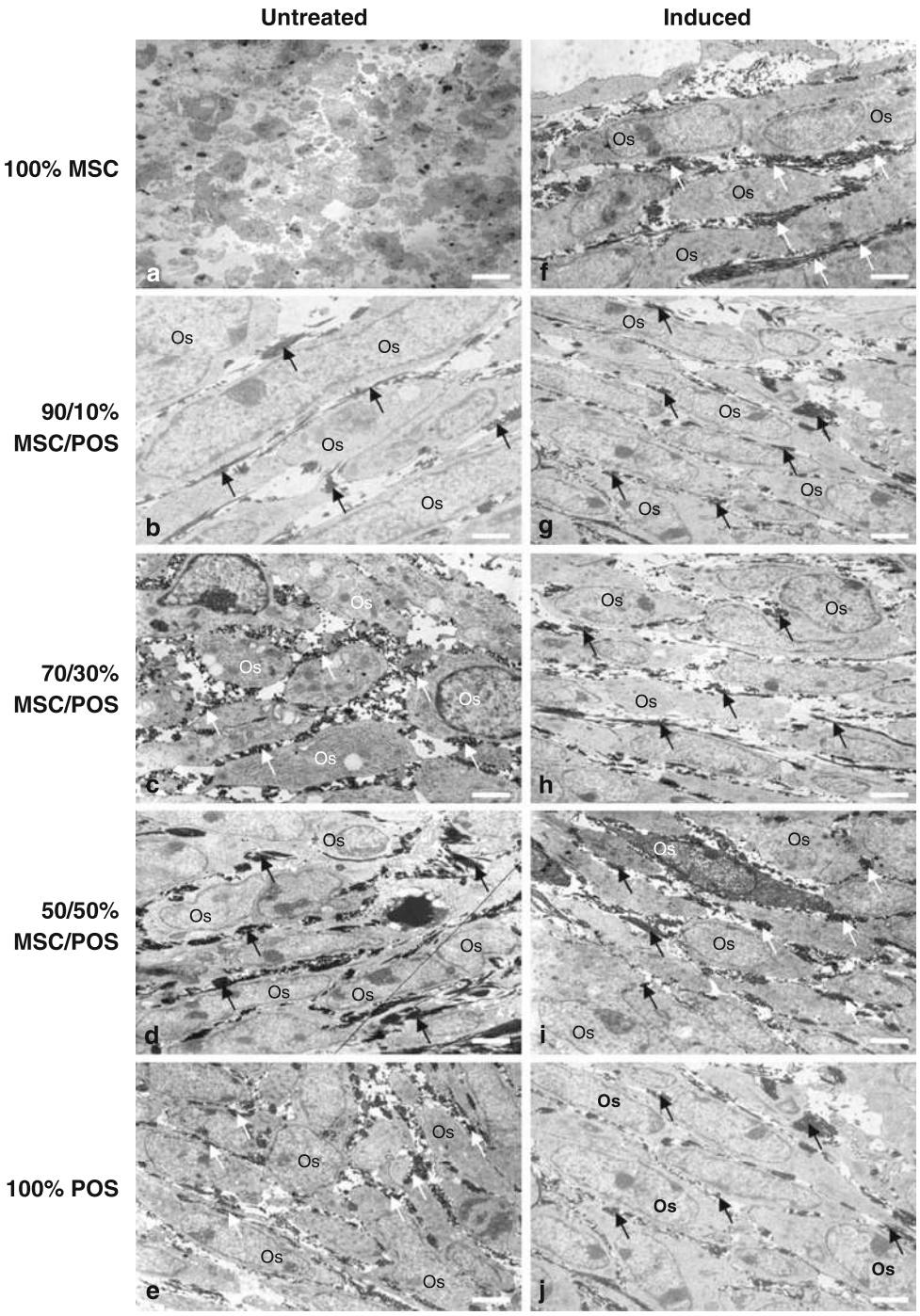



**Fig. 3** High density co-culture; transmission electron microscopy. After 21 days in high-density culture, ultrastructuraly a similar level of osteogenesis (high contents of euchromatin in the cells nucleus, a large number of cell organelles (mitochondria, rough ER, Golgi apparatus), many cell-cell processes and large amounts of thick fibrils of well organised extracellular matrix (*arrows*) was observed in all untreated co-cultures (*b–d*), in untreated primary osteoblast cultures (*e*) as well as in all induced cultures (*f–j*). Untreated MSCs underwent apoptosis, with degeneration of the cells and nucleus, membrane blebbing and formation of apoptotic bodies (*a*). ×6,000, *bar* 1 μm; *Os* osteoblast

*Demonstration of collagen type I and osteocalcin in high density co-cultures treated with and without induction medium by immunoelectron microscopy*

To further characterise the quality of the newly produced extracellular matrix, immunoelectron microscopy was employed and cultures were immunolabeled with antibodies against collagen type I (Fig. 4), which is the most abundant collagen type found in bone matrix and osteocalcin, a bone matrix specific protein (Fig. 5).

High levels of collagen type I expression were confirmed in the untreated and treated primary osteoblast cultures and co-cultures as well as in treated MSCs (Fig. 4; a–i); abundant quantities of gold-particles could be observed directly on the bundles of thick extracellular matrix fibrils (Fig. 4, a–i: arrows in insets). Further, strong labelling for osteocalcin was observed in the untreated and treated primary osteoblast cultures and co-cultures as well as in treated MSCs (Fig. 5; a–i). As expected for osteocalcin, gold-particles were clustered

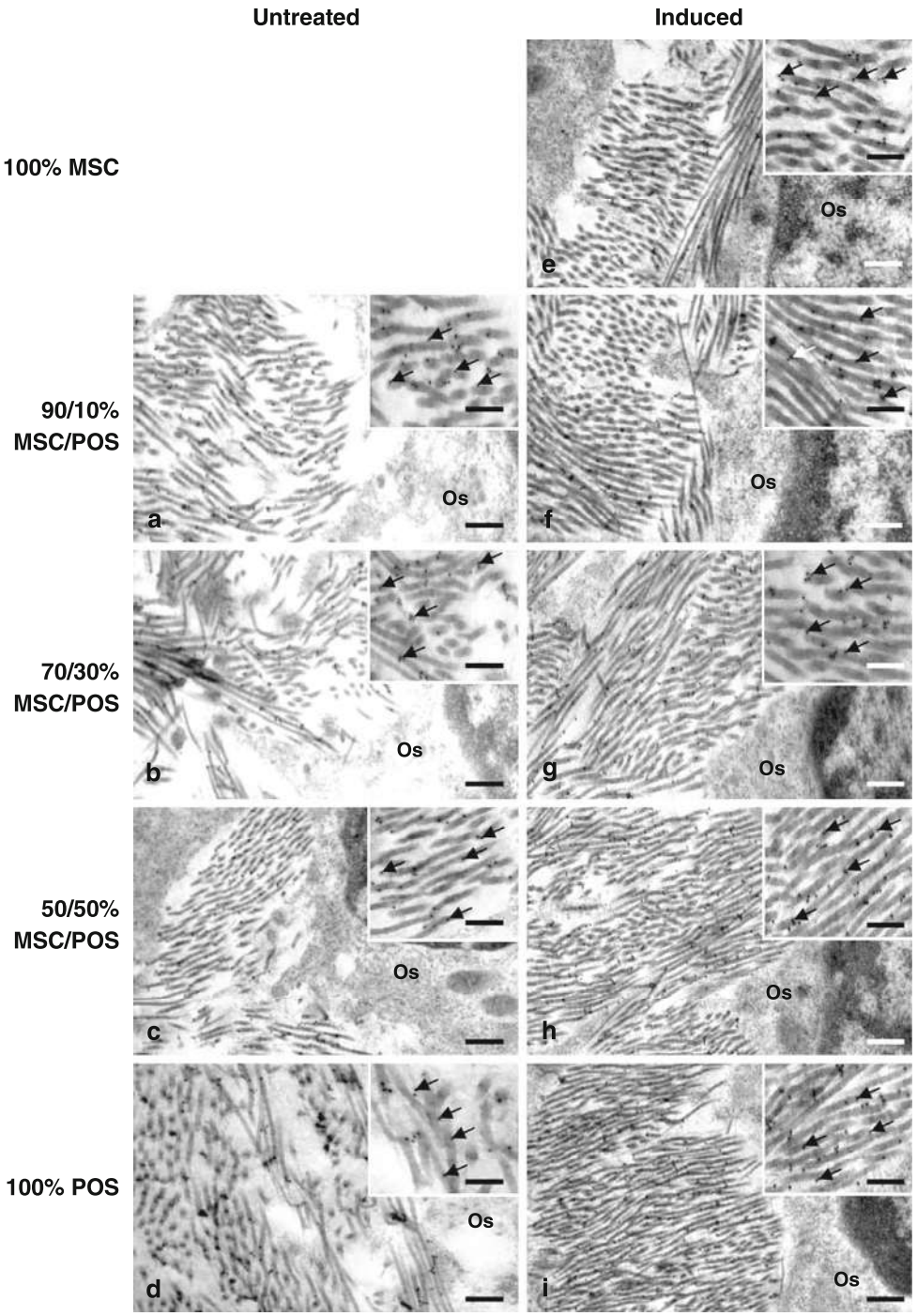



**Fig. 4** High density co-culture; immunoelectron microscopy—Collagen type I. Immuno-labelling of high density cultures after 21 days revealed high amounts of collagen type I in the matrix of untreated and treated primary osteoblast cultures (*d*, *i*) and co-cultures (*a*–*c*, *f*–*h*) as well as in treated MSC cultures (*e*). Secondary gold particle labelled antibody was observed directly on the bundles of thick extracellular matrix fibrils (*insets arrows*). Labelling of untreated MSCs was not performed as apoptosis and necrosis had occurred without matrix formation. ×24,000, *bar* 0.25 μm; *Insets*: 60,000, *bar* 0.1 μm; *Os* osteoblast

together indicating existence of osteocalcin between the mesh of the collagen fibres as is typical for proteoglycan distribution in extracellular matrix (Fig. 5; a–i: arrows in insets).

It was noticeable that quantity and distribution of gold-particles, each representing a collagen type I bound epitope, or an osteocalcin epitope, respectively, were similar between untreated primary osteoblast and untreated co-cultures and in between the different untreated co-cultures. Further, induced primary osteoblast cultures and co-cultures did not have an enhanced labelling compared to untreated cultures. Labelling of untreated MSCs was not performed as apoptosis and necrosis had occurred without matrix formation.

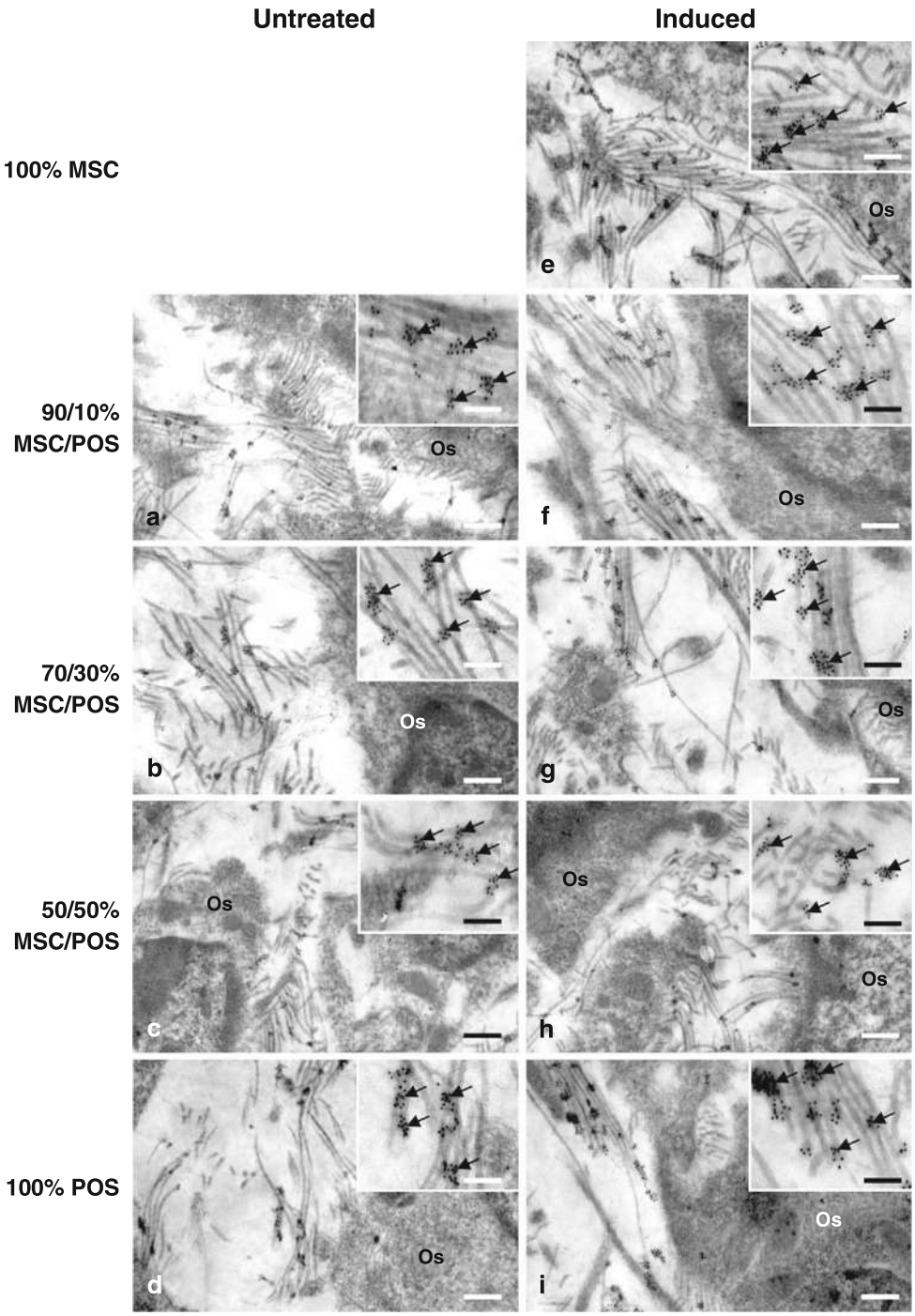



**Fig. 5** High density co-culture; immunoelectron microscopy—Osteocalcin. Immuno-labelling of high density cultures after 21 days revealed high amounts of osteocalcin in the matrix of untreated and treated primary osteoblast cultures (*d*, *i*) and co-cultures (*a*–*c*, *f*–*h*) as well as in treated MSC cultures (*e*). Secondary gold-particle-labelled antibodies clustered together indicating osteocalcin between the mesh of the collagen fibres (*insets*: *arrows*). Labelling of untreated MSCs was not performed as apoptosis and necrosis had occurred without matrix formation. ×24,000, *bar* 0.25 µm; *Insets*: magnification 60,000, *bar* 0.1 µm; *Os* osteoblast

Western blot analysis

*Effects of induction medium and the three dimensional environment on extracellular matrix- and integrin-expression in high density co-cultures*

To confirm the immunomorphological results described above and to quantify the newly produced matrix and analyse the induction of osteogenic signalling pathways in the cells, total protein extracts from high density cultures and co-cultures were resolved by SDS-PAGE, blotted and probed for collagen type I and $\beta$1-Integrin.

In untreated MSC cultures no collagen type I could be detected. In contrast to this, high collagen type I content was detected in treated MSC cultures (Fig. 6a; I). In untreated and treated primary osteoblast cultures similar amounts of collagen type I were detected. Further, in all untreated and treated co-cultures collagen type I was

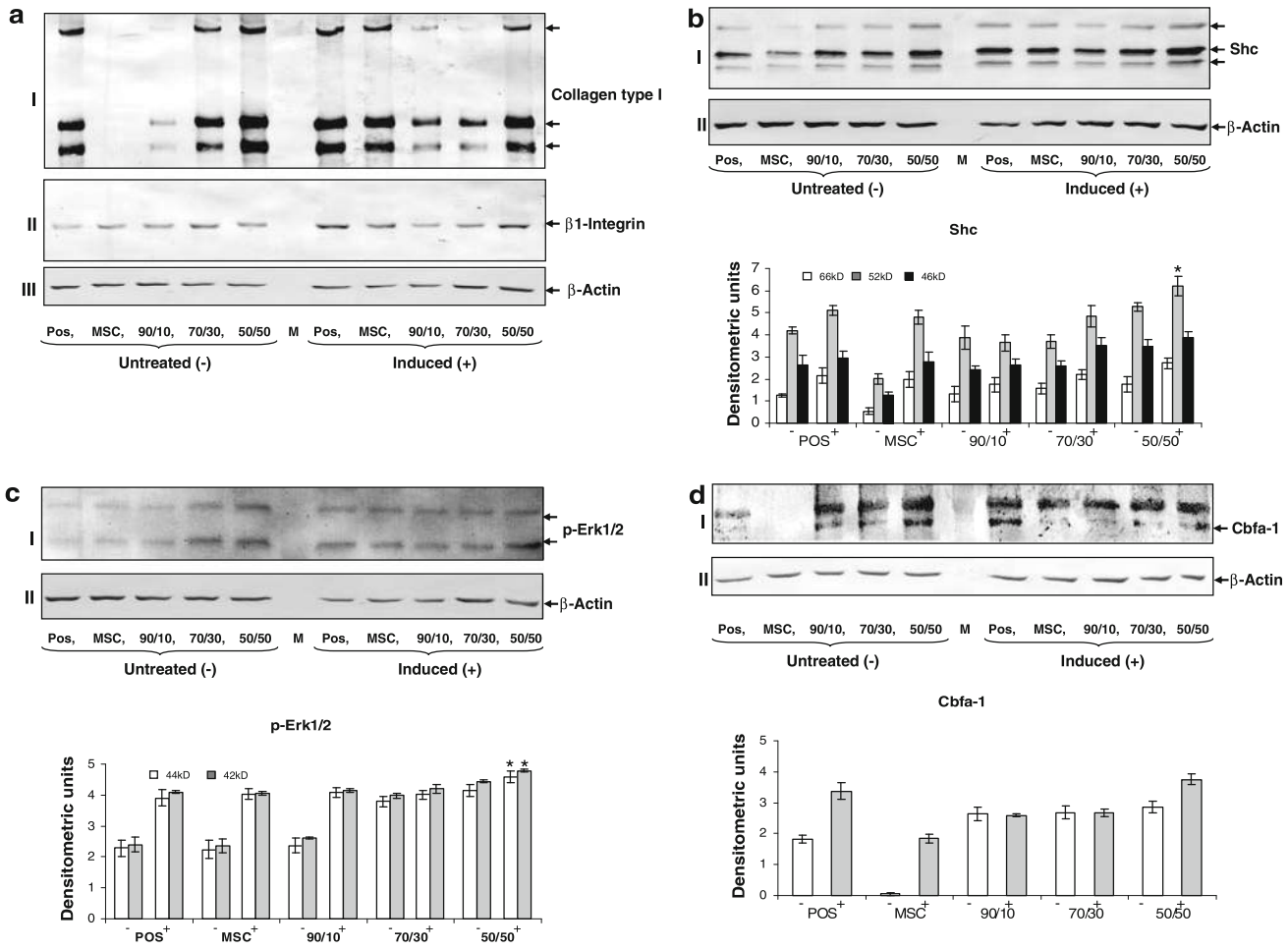



**Fig. 6** Effects of induction medium and the three dimensional environment on extracellular matrix-, integrin-, adaptor protein Shc-, p-Erk1/2- and Cbfa-1-expression in high density co-cultures; high density cultures of mesenchymal stem cells (MSCs), primary osteoblasts (POS) and co-cultures (90/10, 70/30 and 50/50) were incubated with or without growth factors for 21 days. Total cell lysates were analysed by western blot using antibodies that recognise collagen type I (**a**, I), $\beta$1-Integrins (**a**, II), Shc (**b**, I), p-Erk1/2 (**c** I) and Cbfa-1 (**d**, I). Induced MSC cultures demonstrated high protein expression, whereas in untreated MSC cultures none or only weak protein expression was observed. In induced POS cultures and co-cultures enhanced protein expression was demonstrated compared to untreated cultures. In co-cultures protein expression was proportional to the ratio of POS in the co-culture. Protein expression was markedly enhanced with as little as 10% POS in the co-culture; this effect was further promoted through the induction medium. 50% POS significantly enhanced protein expression comparable to pure POS cultures. Densitometric evaluation was performed for Shc (**b**), p-Erk1/2 (**c**) and Cbfa-1 (**d**) and confirmed the above described results. Each experiment was performed in triplicate and mean values and standard deviation are indicated. Values were compared to the control and statistically significant values with $P < 0.05$ were designated by an *asterisk* (*). Expression of the housekeeping gene $\beta$-Actin was not affected. *M* marker

detected. These results confirm the electron microscopy and immunoelectron microscopy findings.

However, immunoblotting further demonstrated that in co-cultures the amount of collagen type I produced was proportional to the ratio of primary osteoblasts in the co-culture. This could be observed for untreated as well as for treated co-cultures (Fig. 6a; I). Interestingly, the amount of collagen type I in co-cultures containing 50% primary osteoblasts was comparable to pure primary osteoblast cultures (Fig. 6a; I). This was observed for untreated as well as for treated co-cultures. In co-cultures containing less then 50% primary osteoblasts (10 or 30%, respectively) significantly lower amounts of collagen type I were detected (Fig. 6a; I). In these co-cultures, treatment with the induction medium enhanced collagen type I production considerably. It was noticeable that in untreated co-cultures containing as little as 10% primary osteoblasts, considerable amounts of collagen type I were detected.

Integrins are adhesion molecules that mediate cell-matrix adhesion and are involved in signal-transduction processes. $\beta$1-Integrin signalling, could be detected in all untreated cultures, with strong, similar levels of expression (Fig. 6a; II). However, in induced cultures $\beta$1-Integrin expression was markedly enhanced in primary osteoblast

cultures and co-cultures containing 50% primary osteoblasts.

*Effects of induction medium and the three dimensional environment on adaptor protein Shc- and p-Erk1/2-expression in high density co-cultures*

Expression of two members of the MAPKinase signalling pathway, the adaptor protein Shc and the extracellular regulated kinase (Erk1/2), were also evaluated, as the MAPKinase signalling pathway has recently been shown to be activated during osteogenic differentiation of MSCs (Jaiswal et al. 2000). In untreated cultures, expression of Shc was equally strong between primary osteoblast cultures and co-cultures (Fig. 6b; I). Although Shc expression was also observed in untreated MSC cultures it was markedly lower (Fig. 6b; I). In all treated cultures and co-cultures strong Shc expression was observed. Densitometric evaluation revealed strongest expression of the 52 kD Shc isoform in all cultures and co-cultures (treated and untreated). Expression of the 52 kD Shc isoform was significantly stronger in co-cultures containing 50% primary osteoblasts compared to the other cultures (Fig. 6b; I: asterisk). Expression of activated Erk1/2 could be observed in all cultures (Fig. 6c; I). In all treated cultures stronger p-Erk1/2 expression in comparison to untreated cultures was observed. Comparing untreated cultures and co-cultures, p-Erk1/2 expression was strongest in co-cultures containing 50 and 30% primary osteoblasts. Interestingly, in treated co-cultures a strong increase in p-Erk1/2 was observed in comparison to treated primary osteoblast cultures. This was statistically significant in the treated co-cultures containing 50% primary osteoblasts (Fig. 6c; I, asterisk).

*Effects of induction medium and the three dimensional environment on expression of the osteogenic specific transcription factor Cbfa-1 in high density co-cultures*

We also evaluated the quantity of the osteogenic transcription factor Cbfa-1 as it is an early marker for osteogenic lineage commitment (Ducy et al. 1997). In untreated MSC cultures no Cbfa-1 expression was detected (Fig. 6d; I). In contrast to this, in treated MSC cultures and in untreated and treated primary osteoblast cultures Cbfa-1 was detected, with enhanced expression in treated primary osteoblast cultures. Further, in all untreated and treated co-cultures Cbfa-1 was detected (Fig. 6d; I). In co-cultures, the detected amount of Cbfa-1 was proportional to the ratio of primary osteoblasts in the co-culture, with markedly stronger expression of Cbfa-1 in induced co-cultures. It was noticeable that in treated cultures, detected amounts of Cbfa-1 were similarly strong between primary osteoblast cultures and co-cultures containing 50% primary osteoblasts.

Osteogenic Induction potential on MSC of secreted substances from POS

To prove that MSC in high density cultures are stimulated by the primary osteoblasts to undergo osteogenesis, MSC high density cultures were treated with the supernatant obtained from primary osteoblast monolayer cultures (Fig. 7). Electron-microscopic evaluation of these MSC high density cultures after 14 days revealed highly active and vital cells embedded in a highly organised, thick fibrillar extracellular matrix (Fig. 7c). High density cultures of MSCs treated with the supernatant from primary osteoblast cultures showed a similar pattern of osteogenic induction as MSC cultures induced towards osteogenesis with a specific osteogenic induction medium (Fig. 7b; c). In contrast to this, in high density MSCs cultures treated only with whole cell culture medium containing 10% FCS, cells demonstrated signs of cell apoptosis and necrosis and consequently no matrix formation was observed (Fig. 7a).

## Discussion

The quest for novel surgical and clinical solutions for the problem of large bone grafts implicates an acute need for more innovative bone replacement techniques. Traditional clinical methods such as allografting are expected to be replaced by new innovations in bone tissue engineering. Although the use of bone grafts is still largely prevalent in the clinic, concerns associated with donor site morbidity and risk of infections has brought more and more focus on tissue engineering approaches (Hangody and Fules 2003; Ross et al. 2000; Silber et al. 2003). In this context mesenchymal stem cells (MSCs) are increasingly studied as a potential source of cells suitable for bone tissue engineering. MSCs essentially overcome the deficiencies associated with the poor regeneration of bone grafts and the limitations of the terminally differentiated osteoblasts (Moioli et al. 2007). MSCs are multipotent cells that exhibit not only chondrogenic and adipogenic potential but also potent osteogenic potential capacities in vitro (Pittenger et al. 1999; Csaki et al. 2007). The osteogenic potential of bone marrow derived MSCs appears to be more prominent compared to the adipogenic and chondrogenic potential of these cells (Kadiyala et al. 1997; Jaiswal et al. 1997). This suggests that a non-homogeneous population of MSCs derived from bone marrow or other sources such as adipose tissue may turn out to be a suitable source of cells for bone tissue engineering.

Several recent studies have demonstrated that after in vivo administration MSCs have the capacity to undergo site

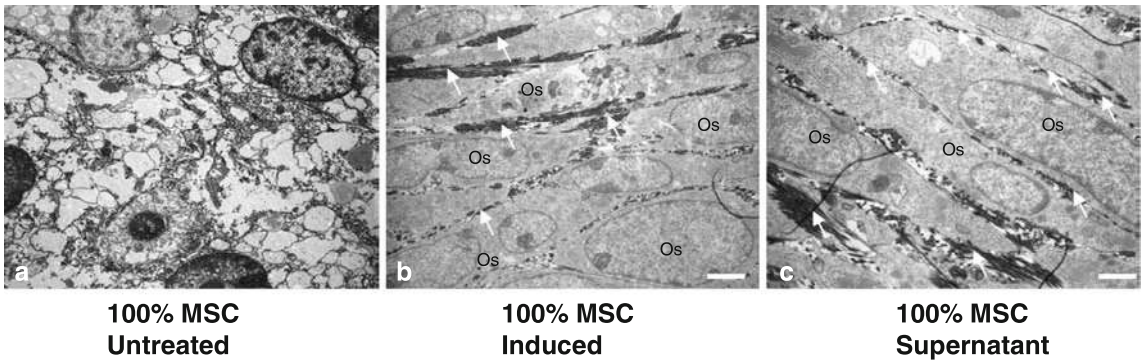



**Fig. 7** MSCs in high density culture treated with the supernatant from primary osteoblast monolayer cultures. Electronmicroscopic evaluation demonstrated induction of osteogenesis in high-density cultures of MSCs by treatment only with supernatant obtained from primary osteoblast monolayer cultures after a period of 14 days in culture (**c**). Induction of osteogenesis was comparable to high-density cultures of MSCs treated with a specific osteogenic induction medium (**b**). In contrast to these observations, no osteogenesis but apoptosis or necrosis was observed in high-density cultures of MSCs treated only with whole cell culture medium containing 10% FCS (**a**). These observations confirm that the primary osteoblasts have an osteogenic inductive effect on the MSCs in a three dimensional environment. ×6,000, *bar* 1 μm; *Os* osteoblast, extracellular matrix (*arrows*)

specific differentiation into chondrocytes, adipocytes, myocytes, cardiomyocytes as well as bone marrow stromal cells and thymic cells (Liechty et al. 2000; Mackenzie and Flake 2001a, b). Furthermore, it has been demonstrated that cultured human MSCs provide a viable model for evaluating the contribution of various factors responsible for their step-wise progression from undifferentiated precursors to secretory osteoblasts capable of matrix mineralisation, and eventually into terminally differentiated bone-resident osteocytes (Jaiswal et al. 1997; Kadiyala et al. 1997). Several studies have shown that direct cell-cell contact with fully differentiated cells may be a critical determinant of mesenchymal stem cell fate (Alexanian and Sieber-Blum 2003; Ball et al. 2004; Richardson et al. 2006). In this study we evaluated the osteogenic induction potential of primary osteoblasts on autologous mesenchymal stem cells in monolayer and three dimensional co-cultures to gain a better understanding of the mechanisms influencing osteogenesis of MSCs for future clinical approaches.

Previous co-culture studies have shown that direct contact is not necessary for the upregulation of osteogenic markers in MSCs (Gerstenfeld et al. 2002, 2003). Further, in the absence of direct cell-cell contacts co-culture of MSCs and chondrocytes result in the up-regulation of osteogenic markers in MSCs (Gerstenfeld et al. 2002, 2003). Furthermore, a study on MSCs with conditioned medium from osteocytes demonstrated that osteocytes produce soluble osteogenic stimulatory signals (Heino et al. 2004) and the authors concluded that osteocytes have an active stimulatory role in controlling bone formation (Heino et al. 2004).

In this study we have clearly demonstrated through PKH membrane labelling, that in monolayer co-culture, primary osteoblasts and MSCs actively make cell-cell contacts, which strongly promotes proliferation of MSCs. We also observed that untreated monolayer co-cultures with primary osteoblasts are sufficient to induce weak osteogenesis (as was confirmed by von Kossa staining). The quantity of nodule formation was proportional to the quantity of primary osteoblasts added. In spite of cell overgrowth, these nodules did not detach but underwent osteogenesis. However, overall osteogenesis in the untreated co-cultures remained lower than in primary osteoblast cultures. This indicates that dynamic cell–cell-contact with fully differentiated primary bone-derived osteoblasts is a strong inducer to promote differentiation of MSCs into osteoblasts.

Our morphological results are consistent with the findings of a recent monolayer co-culture study by Kim and co-workers on rabbit MSCs and rat osteoblasts, where the authors observed calcium deposition and alkaline phosphatase staining in the co-cultures (Kim et al. 2003). These were, however, significantly lower compared to pure osteoblast cultures (Kim et al. 2003). From these observations the authors concluded that there are few synergistic interactions between the cells, otherwise osteogenic induction through the co-culture would have been stronger. However, it must be noted that this study was performed in vitro in monolayer culture and the culture conditions are quite different from the in vivo environment of bone. Therefore, culturing the cells in a three dimensional environment (i.e. in high density co-cultures) might be a decisive factor for stimulating osteogenic cell differentiation. Another issue is that Kim and co-workers co-cultured cells from rabbits with rat cells. It is conceivable that there may be antagonist reactions between cells derived from two different species or that no interaction may exist between cells derived from different species, thus inhibiting osteogenesis.

In stark contrast to the findings of Kim and co-workers, we have shown in this study that, in the three dimensional high density environment osteogenesis is strongly promoted in co-culture. This was demonstrated ultrastructurally using electron microscopy where a similar level of osteogenesis was observed between primary osteoblast cultures compared to co-cultures with regard to cell morphology, cell organelle content and the quantity of newly formed extracellular matrix. We have further demonstrated that the MSCs in high density cultures are stimulated to undergo osteogenesis by the primary osteoblasts; we proved this by stimulating the MSCs only with culture supernatants from primary isolated osteoblasts. Further, ultrastructural characterisation of the co-cultures with immunoelectron microscopy for collagen type I and osteocalcin demonstrated the composition of the newly formed extracellular matrix to be similar. High levels of collagen type I and osteocalcin were detected in primary osteoblast cultures as well as in all co-cultures. This is strongly supportive of the idea that the three dimensional environment, created in high density co-cultures of primary osteoblasts and MSCs, is (a) not only sufficient to induce the MSCs to differentiate towards an osteogenic pathway but (b) further underlines the fact that this system strongly promotes osteogenesis to a level comparable to pure primary osteoblast cultures. Therefore, the three-dimensional environment seems to be of vital importance for promoting osteogenesis and future studies on osteogenesis of MSCs in vitro should account for this important factor.

Integrins are adhesion molecules that mediate cell-cell and cell-matrix adhesion and are involved in signal-transduction processes. Their expression is therefore essential for cell survival. $\beta$1-integrin plays an important role in cell-matrix communication and the maintenance of cell phenotypes in various tissues (i.e. cartilage (Schulze-Tanzil et al. 2001; Shakibaei 1995, 1998; Shakibaei et al. 1997). Moreover, it has been demonstrated that integrin alpha 2 beta 1 and alpha 1 beta 1 are positive regulators of collagen type I synthesis (Riikonen et al. 1995; Shakibaei et al. 1995). In this study we confirmed expression of $\beta$1-integrins in high density cultures. The presence of $\beta$1-integrins underlines the active cell-cell and cell-matrix communication that is taking place in this co-culture system. As expected, $\beta$1-integrin expression was highest in primary osteoblast cultures. However, in co-cultures containing 50% primary osteoblasts $\beta$1-integrin expression was similarly high, indicating an especially active response of the cells in the co-culture to their newly formed bone matrix.

In order to acquire a new phenotype, uncommitted MSCs must undergo proliferative and cell differentiation changes, the two most fundamental biological processes in the lifecycle of cells. One of the signal-transduction pathways that potentially regulates the proliferation and differentiation of MSCs to osteoblasts is the MAPKinase pathway (Jaiswal et al. 2000). Activation of the MAPKinase pathway in other cell types such as neuronal cells, adipocytes, T-cells, and muscle cells promotes cell differentiation (Gredinger et al. 1998; Lowy and Willumsen 1993). The MAPKinase pathway also plays an important role in chondrogenesis (Shakibaei et al. 2001, 2006; Schulze-Tanzil et al. 2004). In this study we evaluated the expression and abundance of activated Erk1/2 during osteogenesis in our co-cultures. P-Erk1/2 are upstream members of the MAPKinase signalling pathway. Recent studies have shown that treatment of MSCs with osteogenic supplements results in a sustained phase of Erk activation that coincides with osteogenic differentiation (Jaiswal et al. 2000). Later during osteogenesis, JNK (c-Jun N-terminal kinase) is further activated, accompanied by sustained extracellular matrix synthesis and elevated osteoid formation and calcium deposition (Jaiswal et al. 2000). It is also known that signalling to the MAPKinase pathway from integrins and epidermal growth factor can be mediated through the adaptor protein Shc (Src homology collagen) activation (Wary et al. 1996). Here, the Shc isoforms of 52 and 44 kD mediate Erk1/2 activation whereas the 66 kD isoform antagonizes Erk activation (Smith et al. 2006). In our high-density cultures we observed upregulation of the two MAPKinase pathway members Erk1/2 and the adaptor protein Shc in primary osteoblast cultures and in induced MSC cultures. We also demonstrated activation of the MAPKinase pathway in our co-cultures. Expression of the Shc 52 kD isoform was especially strong. In fact, co-culture with primary osteoblasts strongly activated members of the MAP-Kinase pathway, compared to the activation observed in pure primary osteoblast cultures. These findings further underscore the importance of the activation of the MAPKinase pathway for osteogenic induction. These observations further highlight the fact that primary osteoblasts must be actively involved in signalling osteogenic lineage commitment to the MSCs.

Finally, we observed activation of the osteogenic specific transcription factor Cbfa-1. Cbfa-1 is the earliest and most specific marker of osteogenesis (Ducy et al. 1996; 1997 Ducy 2000). In cell culture, Cbfa-1 acts as an activator of transcription and can induce osteoblast-specific gene expression in fibroblasts and even myoblasts (Ducy et al. 1997). It has been shown that mechanical stress regulates Cbfa-1 activation and favours osteoblast differentiation through the activation of the MAPKinase signal-transduction pathway and Ras/Raf-dependent Erk1/2 activation, independent of p38 MAPK signalling (Kanno et al. 2007). In this study, Cbfa-1 activation was observed in control and induced primary osteoblast cultures and co-cultures as well as in induced MSC cultures. There was a relationship between Erk1/2 activation and Cbfa-1 activation, emphasising the close connection between the MAPKinase pathway

and the osteoblast-specific transcription factor Cbfa-1. In the co-cultures Erk1/2 and Cbfa-1 activation was significantly higher in co-cultures containing 50% primary osteoblasts, comparable to primary osteoblast cultures, suggesting even a possible synergistic effect on osteogenesis through high density co-cultures.

In conclusion, this study has demonstrated that changes in cell proliferation, matrix production and protein expression are induced in MSCs when they are co-cultured with primary osteoblasts. Our results suggest that the osteogenic differentiation of MSCs is not only aided through close cell-cell contacts but also it is enhanced by the three dimensional environment in the co-cultures. Furthermore, we have demonstrated enhanced p-Erk1/2 and Cbfa-1 expression in co-cultures containing 50% primary osteoblasts in comparison to pure primary osteoblast cultures suggesting not only an inductive but also an additive or perhaps even synergistic effect of primary osteoblasts on the osteogenic differentiation of MSCs in co-culture.

Therefore, the high density co-culture system adopted in this study might provide a physiologically relevant model for future studies on the differentiation of MSCs and the process of osteogenesis. New information gained using this model system may provide new ways to control and regulate osteogenesis of MSCs and facilitate future clinical applications of MSCs in tissue engineering.

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