Dexamethasone Modulates BMP-2 Effects on Mesenchymal Stem Cells In Vitro

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ABSTRACT: Dexamethasone/ascorbic acid/glycerolphosphate (DAG) and bone morphogenic protein (BMP)-2 are potent agents in cell proliferation and differentiation pathways. This study investigates the in vitro interactions between dexamethasone and BMP-2 for an osteoblastic differentiation of mesenchymal stem cells (MSCs). Bone marrow-derived human MSCs were cultured with DAG (group A), BMP-2 + DAG (group B), and DAG + BMP-2 combined with a porous collagen I/III scaffold (group C). RT-PCR, ELISA, immuncytochemical stainings and flow cytometry analysis served to evaluate the osteogenic-promoting potency of each of the above conditions in terms of cell morphology/viability, antigen presentation, and gene expression. DAG induced collagen I secretion from MSCs, which was further increased by the combination of DAG + BMP-2. In comparison, the collagen scaffold and the control samples showed no significant influence on collagen I secretion of MSCs. DAG stimulation of MSCs led also to a steady but not significant increase of BMP-2 level. A DAG and more, a DAG + BMP-2, stimulation increased the number of mesenchymal cells (CD105+/CD73+). All samples showed mRNA of ALP, osteopontin, Runx2, Twist 1 and 2, Notch-1/2, osteonectin, osteocalcin, BSP, and collagen-A1 after 28 days of in vitro culture. Culture media of all samples showed a decrease in Ca^{2+} and PO_4^{2-} concentration, whereas a collagen-I-peak only occurred at day 28 in DAG- and DAG + BMP-2-stimulated bone marrow cells. In conclusion, BMP-2 enhances DAG-induced osteogenic differentiation in mesenchymal bone marrow cells. Both agents interact in various ways and can modify osteoblastic bone formation. J Orthop Res 26:1440–1448, 2008

Keywords: mesenchymal stem cell; bone morphogenic protein; dexamethasone; osteoblast

Several factors are known to be potent inducers of bone formation in osteoprogenitor cells both in vivo or in vitro. Here, some members of the transforming growth factor (TGF)β superfamily such as BMP-2 and BMP-7 have shown promising clinical results in fracture healing, spinal fusion, and implant fixation.[1–3] In addition, dexamethasone (D), ascorbic acid (A) and β-glycerolphosphate (G) have been shown in many in vitro experiments to induce osteoblast differentiation of mesenchymal progenitor cells.[4–6] Furthermore, some data also indicate that these osteogenic supplements may also have mitotic activity.[7]

Besides the potency of biomineralization, osteoblasts are characterized by an expression of typical markers such as alkaline phosphatase (ALP), osteopontin, osteonectin, osteocalcin, osteoprotegerin, bone sialoprotein (BSP), collagen I, nuclear factor-kappaB ligand (RANKL), Runx 2, core binding factor (Cbfa) 1[8] and osterix (Osx).[9–13]

After specific receptors binding, BMP-related effects are mediated by different signaling pathways including the Ras/MAPK system,[14] different Smad proteins,[15] Ca^{2+}, cAMP, promyelocytic leukemia zinc finger protein (PLZF),[16] the Runx/Cbfa1 pathway,[14,17–19] and the Wnt/β-catenin system.[20] Especially BMP-2, -6, -7, and -9 are potent to induce osteoblast differentiation and produce a distinct set of molecular fingerprints during osteogenic differentiation.[21]

However, it is not clear that the combination of BMPs and DAG will enhance or accelerate the in vitro differentiation of osteoblasts and if these factors are stage limiting. Several authors also reported that biomaterials such as tricalciumphosphate (TCP), hydroxyapatite (HA),[22] and collagen I[23–25] have osteoblast-promoting potency. Beside the physico-chemical properties of these biomaterials, surface parameters such as porosity, microarchitecture, and topography significantly influence cellular adherence, migration, proliferation, differentiation, and survival of osteoprogenitor cells.[22] In this study, the in vitro osteogenic potential of BMP-2, DAG, and collagen I/III are investigated by utilizing a human bone marrow cell culture.

MATERIALS AND METHODS

Cell Culture

Human bone marrow stromal cells obtained from the posterior iliac crest of three different donors (46-year-old male, and 20- and 28-year-old female individuals) were isolated, cultured, and expanded in vitro up to 12 days (1st passage) as described by previous protocols.[26,27] All volunteer donors had given written consent according to the Declaration of Helsinki in its present form.

Cell culture conditions were DMEM-low glucose media, 20% fetal calf serum (FCS)-gold (all agents PAA Laboratories, Cölbe, Germany), 1% penicillin/streptomycin/L-glutamine (PAA) in tissue culture polystyrene flasks in 5 Vol.% CO_2 at 37°C. Medium was exchanged twice a week. Adherent cells, judged 80%–90% confluent by phase contrast microscopy, were

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detached and passaged using a 0.05% trypsin/0.02% EDTA solution. A total of 5,000 human bone marrow cells/1.9 cm^2 were seeded into each culture dish, and these cell cultures were stimulated as follows for a total follow-up of 28 days:

- Group A: DAG (10 μM dexamethasone, 50 mM L-ascorbic-2-phosphatate, 10 mM β-glycerolphosphate; Sigma, Taufkirchen, Germany)
- Group B: DAG + 50 ng BMP-2/mL media (Sigma, Taufkirchen, Germany)
- Group C: DAG + BMP-2 + 1 cm × 1 cm of a three-dimensional (3D), procine collagen I/III mesh (Col) (DAG + BMP-2/Col; ACI-Maix™, Fa. Matricel GmbH, Herzogenrath, Germany)

Human bone marrow cells without DAG, BMP-2, and collagen incubated under the same conditions served as controls. All experiments were carried out in triplicate. Episcopic light microscopy was performed every 2nd day to evaluate morphology and proliferation of the cells.

Immuncytochemical Stainings

Before and 28 days after in vitro stimulation, cell monolayers were stained against the following factors and antigens as described previously.[26] Alkaline Phosphatase (ALP; Blue Alkaline Phosphatase Substrate Kit III, #SK-5300, Vector Laboratories, Burlingame, CA), Receptor-activator of NFkB-ligand [RANKL; rabbit-anti, 1:100/PBSTA (10 μL/mL), Santa Cruz SC-9073, Santa Cruz Biotechnology, Santa Cruz, CA], Twist (rabbit-anti, 1:200/PBSTA, Santa Cruz SC-15393, Santa Cruz Biotechnology, Santa Cruz, CA). Signal was enhancement by an anti-rabbit-IgG/biotin secondary antibody system (anti-rabbit-IgG/biotinylated, avidin-biotin-complex, Vector; +DAB, Sigma). At follow-ups, bone marrow cells were morphologically analyzed using phase-contrast microscopy (Axiovert 200, Zeiss, Jena, Germany) supported by a computer picture analysis system (Axiovision, Zeiss). For semiquantitative analysis of the antigen expression by polystyrene adherent cells, the following score was used: no cells: 0; single cells: 1; sub-confluent monolayer of + cells (<10%–59%): 2; confluent monolayer of + cells (>60%): 3.

Flow Cytometry

To evaluate effects of the different culture supplements (DAG, DAG + BMP-2) onto the survival of human bone marrow cell, a quantitative measurement of both vital and dead cells was performed by flow-cytometric analysis. After detachment of adherent cells by vortexing supported by incubation in 0.05% trypsin/0.02% EDTA for 1 min, a volume of 1 mL cell suspension was stained using DRAQ 5 (Biostatus Lim, LE, UK) at 10 μM for DNA staining to quantify cell viability and Propidiumiode at 10 μg/mL (Sigma, St. Louis, MO) for dead cell exclusion. A total volume of 240 μL cell suspension was analyzed on an FC500 dual laser flow cytometer (Beckman Coulter, Krefeld, Germany) and viable cell concentration determined. Because it was not possible to remove cells from the collagen matrix and get reliable results, this group was not considered for flow cytometry. Human bone marrow cells without DAG, BMP-2 of Col I exposure served as controls. Furthermore, expression of CD73 (CD73-PE, Pharmingen #550257, BD Pharmingen, San Diego, CA), CD105 (CD105-FITC, Amcel #326040), CD34 (CD34-ECD, Immunotec #IM 2709, Immunotec, Marseille, France), CD 45 (CD45-PC7, Beckman Coulter #IM3548, Beckman Coulter, Krefeld, Germany) was quantified by flow cytometry (20 μL AK-Mastermix for each probe, 30-min incubation period, control beads: BD™ CompBeads/Anti-Mouse IgG 51-90-900-1229, Bector Dickinson, Heidelberg, Germany).

RT-PCR

For mRNA analysis, the adherent cells were removed from culture dishes supported by 0.05% trypsin and resuspended in each 350 μl RLT buffer (Fa. Quiagen, Germany) supplemented by 1:100 14.3 M β-mercaptoethanol. One-step RT-PCR was performed using a thermal cycler (Eppendorf Mastercycler Gradient, Eppendorf AG, Hamburg, Germany). The reaction mixture (25 μL) contains 3 μL of human RNA, 1 μL 10 mM dNTP-Mix, 1 μL recombinant Rnasin™/ribonuclease inhibitor 1:4 (Promega, Madison, WI), 20 pM of each primer, 1 μL enzyme mix, 5 μl RT-buffer, 5 μL Q-solution, and 7 μL RNAse free *aqua* (Quiagen, Hilden, Germany). For transcription and amplification, we used an enzyme mix containing Omnicript™ Reverse transcriptase, Sensiciript™ Reverse Transcriptase, and HotStar*Taq* DNA polymerase [OneStep RT-PCR Kit (100), Fa Quiagen, Hilden, Germany]. The thermal cycle conditions used were as follows: 30 min at 50°C (reverse transcription), 15 min at 95°C (denaturation) followed by RT-PCR cycling of 95°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 60 s (extension), and lastly a final extension for 60 s at 72°C. RT-PCR was performed 35 cycles. GAPDH served as negative control. RT-PCR products were combined and resolved in a 2% agarose gel stained with ethidium bromide. Documentation and semiquantitative evaluation was performed using a gel documentation system and software (including a real-time camera with ethidium bromide filter combined with a Alpha-DigiDoc™ RT software, Alpha Innotec, San Leonardo, CA). Oligonucleotide primers: Osteonectin (ON): 5′-ggctcaagaacgtcctggt-3′/3-′ctgcttgatgccgaagcag-5′ (NM_003118), osteocalcin (OC): 5′-agtccagcaaaggtgcagc-3′/3-′ggccgtagaagcgccgat-5′ (NM_199173), osteopontin (OP): 5′-gaacgactctgatgatgtag-3′/3′-gctcattgcctccatcattg-5′ (NM_000582), RANKL: 5′-cagagcgcagatggatcct-3′/3′-gtaccaagaggacagactca-5′ (NM_003701), twist1: 5′-ggcaagctgcagctatgtg-3′/3′-gacctcttgagaatgcatgc-5′ (NM_000474), twist2: 5′-gacattgtcatggtcatcttg-3′/3′-cttgaaacaatcatgcagagg-5′ (NM_057179), notch1: 5′-caagcaagttctgagagcca-3′/3′-tgcaaattaatccgcgtgcg-5′ (NM_017617), notch2: 5′-ctgccttccagaaacagtga-3′/3′gtgccacgcatagtctctc-5′ (NM_203458), runx2: 5′-ccttcaaggtggtagccct-3′/3′-gcctggggtctgtaatctg-5′ (NM_004348), collagen 1A1: 5′-gatggctgcacgagtcaca-3′/3′-gccagatggcaaggcttct-5′ (NM_000088), bone sialoprotein (BSP): 5′-tggggtctttaagtacaggc-3′/3′-tttgttatatccccagccttc-5′ (NM_004967), alkaline phosphatase (ALP): 5′-gcttcagaagctcaacacca-3′/3′-cacgatgcctttcaccacg-5′ (NM_013227), and GAPDH: 5′-ctcaagatcatcagcaatgcc-3′/3′-gatggtacatgacaaggtgc-5′ (NM_002046).

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

To quantify the local amount of BMP-2 and collagen I in culture media during cell cultivation, a sandwich-ELISA was performed (Quantikine, R&D Systems, Minneapolis, MN) as described by Bae et al.[28] on days 0, 14, and 28. For photometric evaluation, we used a microplate reader (Cary 50 Microplate Reader, Varian, Darmstadt, Germany). The concentrations of Ca^{2+} and PO_4^{2-} within the culture media were evaluated photometrically using standard kits. Ca^{2+} detection is on an o-cresolphthalein-complexion reaction (Calcium CPC FS, DiaSys Diagnostic Systems, Holzheim, Germany), whereas PO_4^{2-} was detected photometrically by an ammoniummolybdat/sulfuric acid (Phosphat FS, DiaSys Diagnostic Systems).

The concentration of both ions were calculated by the rule of three using a standard curve as described by the manufacturer.

Statistics

The Student's t-test (one-sided, paired) was used for statistical analysis. $p < 0.01$ was rated highly statistically significant and $p < 0.05$ statistically significant, whereas $p > 0.05$ showed no significance. The average values (X) and standard deviations (SD) served as descriptive parameters.

RESULTS

Cell Proliferation and Morphology

All three cell cultures (DAG+, DAG + BMP-2, DAG + BMP-2/Col) showed progressive morphological signs of an osteoblastic differentiation and also of biomineralization with further cultivation. A local mineral deposit was first noticed after a period of 7 days of in vitro stimulation. There were crystal structures at different regions within the cultures which showed an average diameter of 5–300 μm, and 14 days after stimulation these crystals become more and more confluent. In contrast to these findings, cells of negative controls without osteogenic stimulation showed a spindle-shape morphology without any signs of mineralization (Fig. 1a).

Cell Viability

There were differences on the number of dead cells between controls, DAG-, and DAG + BMP-2-stimulated cultures. The highest amount of dead cells was found in DAG + BMP-2-stimulated cultures (X: 12.1%, SD: 5.4), whereas controls showed 10.98% (SD: 7.6) dead cells. There were less but not significant dead cells in DAG+ cell cultures (X: 6.36%, SD: 0.57, $p_{\text{DAG vs. DAG+BMP2}}$: 0.089) compared to both other groups.

Number of Mesenchymal and Hematopoietic Cells

There was a significant influence on the number of mesenchymal cells in dependency of supplementation with DAG or DAG + BMP-2 (Fig. 2). The proportion of CD105+/CD73+ mesenchymal cells/all viable cells decreased from initially 88.5% to 61.1% in controls at the end of follow-up. A DAG and more, a DAG + BMP-2, stimulation increased the number of CD105+/CD73+ cells, whereas inverse effects were seen for the CD105−/CD73− non-mesenchymal cells (X_{control}: 30.9% vs. X_{DAG}: 20.2% vs. $X_{\text{DAG+BMP2}}$: 4.6%), but these differences were not significant compared to controls ($p > 0.05$). However, the least number of CD105−/CD73− cells was found at day 0 before stimulation (0.9%). Compared to day 0, the decrease of CD105+/CD73+ cells was significant for DAG+ BMP-2 cell cultures ($p = 0.017$) at day 28. We

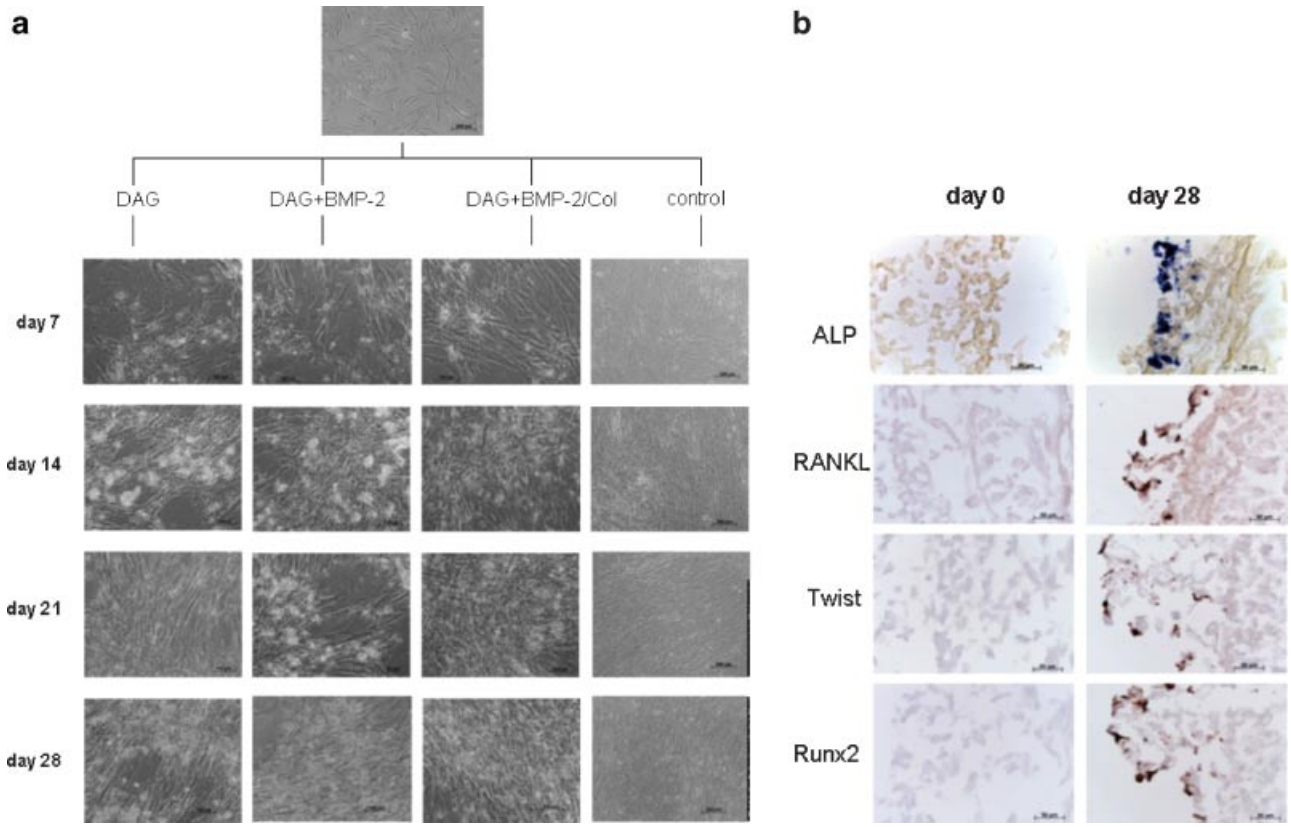

Figure 1. Morphological and immunohistochemical findings of human bone marrow-derived cells after stimulation with DAG, DAG + BMP-2, and DAG + BMP-2/Col. (a) Episcopic light microscopy showed signs of an osteoblastic differentiation after 7 days in vitro with a cuboid cell type. From days 14 up to 28, there were significant signs of biomineralization in all stimulated cultures compared to controls. (b) Human bone marrow-derived cells differentiated osteoblastic during cultivation onto the porous collagen I/III scaffold and migrated up to 100 μm in the 3D collagenous fibrous network texture.

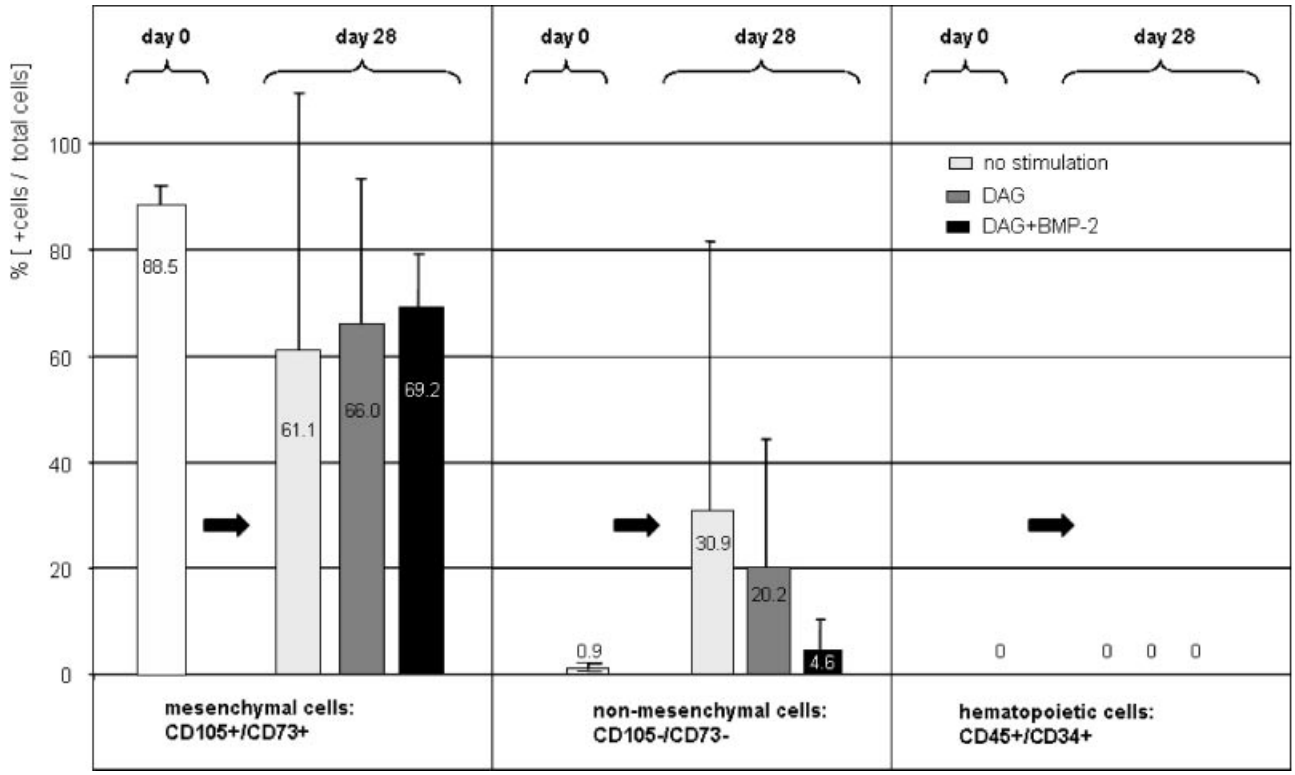

Figure 2. Flow cytometer showed no CD34+/CD45+ cells before osteogenic stimulation of the passaged bone marrow cultures. There was a slight increase of mesenchymal CD105+/CD73+ cells under osteogenic stimulation and a corresponding reduction of non-mesenchymal CD105−/CD73− cells in dependency to the stimulus.

found no hematopoietic CD45+/CD34+ cells before and after stimulation in any of the samples.

Expression of Osteoblastic Proteins

The semiquantitative evaluation of adherent cells by immunocytochemical stainings showed differences in the expression of osteoblast-specific antigens between the groups (Fig. 3). In all cultures, we found an increased number of ALP+ cells after 28 days compared to day 0.

At follow-up, both DAG-stimulated and control cultures expressed increased numbers of RANKL+ cells compared to day 0. DAG + BMP-2-stimulated bone marrow cells showed the highest number of RANKL+ cells. Runx2 was expressed by a dense monolayer in DAG and DAG + BMP-2-stimulated bone marrow-derived cells compared to controls. At day 0, there was no expression of Twist, whereas at day 28, we found a Twist-expression in controls but more Twist + cells in DAG and DAG + BMP-2 cultures.

In contrast to the condition at 12 h after cultivation, the adherent cells on the porous collagen I/III scaffold expressed all osteoblastic markers tested (ALP, Runx2, Twist, and RANKL) after 28 days in vitro (Fig. 1b).

mRNA-Expression of Osteoblastic Genes

With the exceptions of RANKL and OP, there were no qualitative differences in the expression of osteoblastic genes between the different groups at days 0 and 28 in vitro. The study groups and the control expressed ALP, OP, Runx2, Twist 1 and 2, Notch 1 and 2, ON, OC, BSP, and Collagen IA1 after 28 days in vitro. However, at day 0, we found only a very weak RANKL signal and an OP band in two of three donors by RT-PCR in human bone marrow cells. All DAG + BMP-2-stimulated cell cultures (three bone marrow donors) expressed RANKL and also two DAG-stimulated cell cultures out of three bone marrow donors expressed RANKL after 28 days in vitro. In contrast, we found no RANKL expression in unstimulated controls (Fig. 4).

BMP-2, Ca^{2+}, PO_4^{2-}, and Collagen I Concentration

BMP-2

There were significant differences in BMP-2 concentration in dependency of the media supplements and the cultivation time in humane MSC cultures. In controls, the BMP-2 concentration decreased significantly during cultivation from $X_{day\ 4}$: 1,550 pg/mL (SD: 132.3) to $X_{day\ 28}$: 113 pg/mL (SD: 157.2) ($p_{day\ 4/day\ 28} = 0.005$), whereas we found a significant increase of BMP-2 amount in DAG-stimulated cultures from $X_{day\ 4}$: 938.5 pg/mL (SD: 250) to $X_{day\ 28}$: 1,307 pg/mL (SD: 378.6) ($p_{day\ 4/day\ 28} = 0.036$). An initial BMP-2-peak of 1,710 pg/mL (SD: 190.5) at day 4 was followed by a highly significant drop down to 1,100 pg/mL (SD: 173.2) at day 14 in DAG + BMP-2-stimulated hMSC cultures ($p_{day\ 4/day\ 14} = 0.00013$). At day 28, these cultures showed no significant changes in BMP-2 concentration compared to day 14. In contrast, the cultures which were exposed to DAG + BMP-2/Col showed a slightly

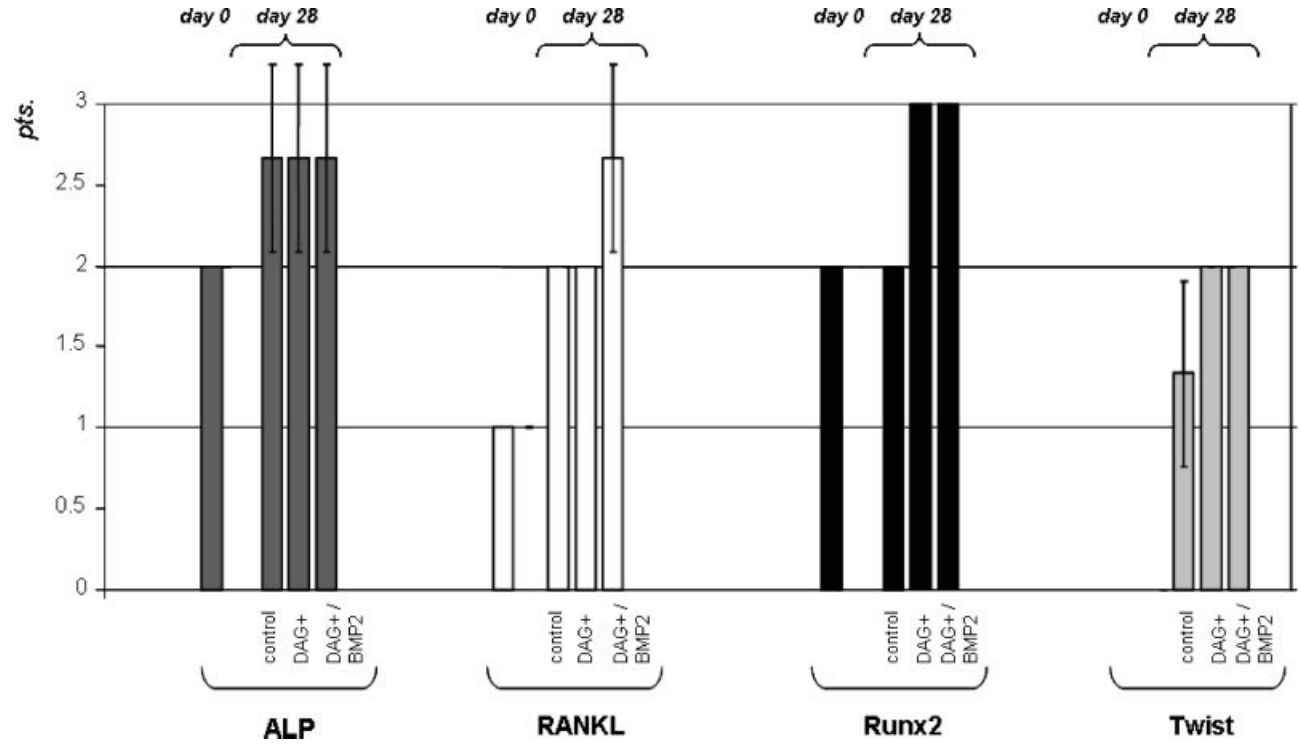

Figure 3. Both BMP-2 and DAG lead to a significant increase of Runx2 expression, whereas BMP-2 stimulation increased RANKL compared to cell cultures which were exposed to DAG only.

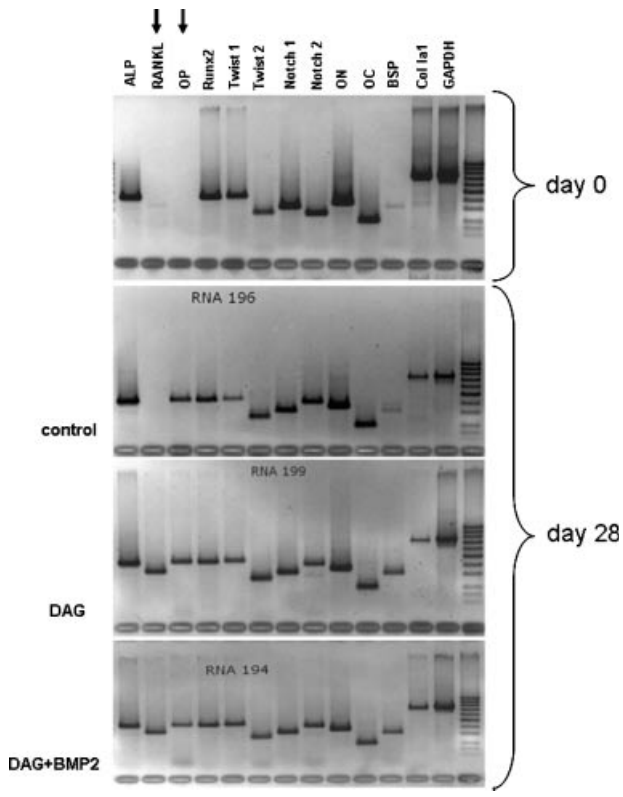

Figure 4. mRNA expression of different osteoblastic markers in vitro. ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; ON, osteonectin; Col IA1, collagen IA1; BSP, bone sialo protein; RANKL, receptor of activator of nuclear factor-kappa-B ligand.

decrease from day 4 (X: 1,140 pg/mL, SD: 519.6) to day 14 (X: 133.3 pg/mL, SD: 251.7) followed by an increase of BMP-2 at day 28 (1,533.3 pg/mL, SD: 404.1). However, these effects were not significant.

Comparing the BMP-2 concentration between the different groups, BMP-2 was significantly increased in controls at day 4 compared to DAG-stimulated cultures ($p = 0.029$) but significantly decreased at day 28 ($p = 0.036$). Furthermore, BMP-2 was increased in the DAG + BMP-2 group compared to DAG + cultures at day 4 ($X_{DAG+BMP\text{-}2}$: 1,710 pg/mL, SD: 190.5 vs. X_{DAG+}: 938.5 pg/mL, SD: 250) but showed the same level at day 14 ($X_{DAG+BMP\text{-}2}$: 1,100 pg/mL, SD: 173.2 vs. X_{DAG+}: 1101 pg/mL, SD: 726.7).

It was evident that at the end of follow-up, the BMP-2 concentration showed comparable values without significant differences in all groups ($X_{control}$: 1,130 pg/mL, SD: 157.2 vs. X_{DAG}: 1,307 pg/mL, SD: 378.6 vs. $X_{DAG+BMP\text{-}2}$: 1,200, SD: 86.6 vs. $X_{DAG+BMP\text{-}2/Col}$: 1,533.3, SD: 404.1). Figure 5 shows an overview of the BMP-2 and collagen I expression by human bone marrow cells under different stimuli within 28 days in vitro.

Ca^{2+} and PO_4^{2-} Amount

Both Ca^{2+} and PO_4^{2-} concentrations in culture media showed a similar curve at 4, 14, and 28 days. In all cultures, the concentration of both molecules decreased during cultivation. As shown in Figure 6, a supplementation of DAG with BMP-2 led to higher initial values at cultivation day 4 ($Ca^{2+}{}_{DAG+BMP\text{-}2}$: 215.3 vs. $Ca^{2+}{}_{DAG}$: 103.3 mmol/l, and $PO_4^{2-}{}_{DAG+\ BMP\text{-}2}$: 116.2 mg/dl vs.

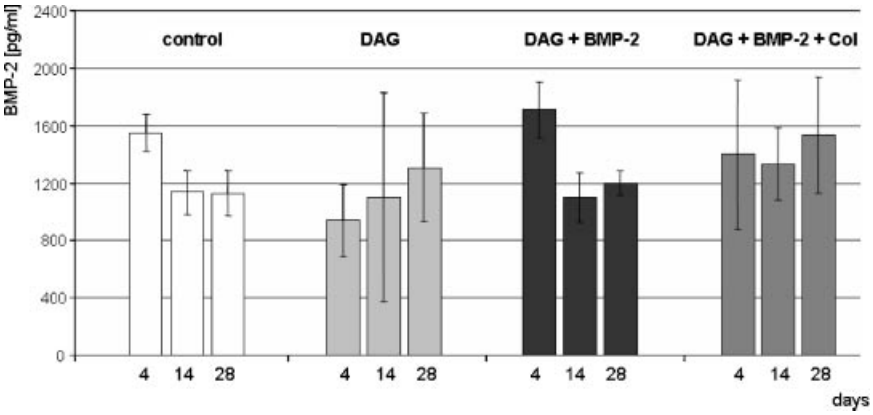

Figure 5. BMP-2 concentration in culture media at different cultivation days. Human MSC-cultures were exposed without DAG (control), with DAG only, with DAG + BMP-2 and with DAG + BMP-2/Col.

$PO_4^{2-}{}_{DAG}$: 76.6 mg/dl). The presence of a collagen I/III scaffold led to low concentrations of Ca^{2+} and also PO_4^{2-} within the culture media.

Collagen I Secretion

It was evident that DAG- and DAG + BMP-2-stimulated cell cultures showed collagen I peak at day 28 compared to controls and to bone marrow cells which were incubated with additional collagen I/III carrier.

DISCUSSION

In this study, we showed that DAG, DAG + BMP-2, and DAG + BMP-2/Col induce an osteoblastic in vitro differentiation of human bone marrow-derived MSCs. During cultivation, there were no morphological differences between the different cell cultures under an episcopic light microscopy. Our data contradicted the results found by Jorgenson et al.[29] who reported that both dexamethasone and BMP-2 can be used as osteoblast inducers but influence cellular differentiation to a different phenotype. We confirm the results of other investigators who showed osteoblastic differentiation of MSCs under BMP-2 supplemented collagen I-based scaffolds.[30–32] However, it remains unclear if dexamethasone can selectively induce apoptosis of some populations of bone marrow cells which were thought to have poor differentiation capability as suggested by Oshina et al.[33] or if glucocorticoids fail to induce terminal osteoblast differentitation.[34]

Our results also corresponded to Hong et al.[35] who reported an early expression but a late secretion and deposition of collagen I in osteoblast cultures. We found significant peaks of collagen I in DAG and DAG + BMP-2-stimulated cultures but not in controls at day 28, whereas the collagen I levels in culture media showed no significant differences at days 4 and 14. In addition, Gerstenfeld et al. reported a decrease in collagen I synthesis after day 12 in vitro, but a sixfold total collagen I increase in the cell layers from days 12 to 30,[36] which corresponds to our findings. Although glucocorticoids inhibit collagen I synthesis in osteoblast (e.g., by induction of collagenase expression[37]), some data also show that the finding of increased levels of collagen I in DAG-supplemented cultures may be based on the presence of ascorbic acid. Several studies have shown that ascorbic acid increases proline hydroxylation in the intracellular procollagen pool, stimulates the cleavage of type I collagen propeptides, and also increases the rate of procollagen secretion from cell layers to culture medium.[38] Also, β-glycerolphosphate promotes biomineralization and expression of osteoblastic proteins such as collagen I and ALP.[39]

Our data indicate that DAG + BMP-2 induce high levels of RANKL in bone marrow cells, whereas the Runx2 expression was strongly promoted by both DAG and DAG + BMP-2 compared to controls. Jorgenson et al.[29] showed that the ALP activity was increased by dexamethasone but not by BMP-2, and procollagen type I (P1NP) production was decreased by dexamethasone while BMP-2 had no effect on P1NP levels. Koch et al.[40] demonstrated that BMP-2 is a potent stimulator of an osteoblastic gene expression in the absence of dexamethasone. Besides species-related and individual differences, it is evident that BMP-2 regulates not only osteoblast differentiation but also influences other pathways of different human cell types.[41–44] It also should be considered that glucocorticoids influence the recruitment and differentiation of other bone cells such as osteoclasts, as shown by Defranco et al.,[45] and also that a large number of genes respond to BMP-2 stimulus.[46] In our study, we did not evaluate these bone-resorbing cells, but we did observe an increased number of dead cells in DAG + BMP-2-stimulated cultures (X: 12.1%) compared to DAG+ (6.36%) and control cultures (10.98%). As shown by Rai et al.,[47] BMP-2 promotes osteoblastic differentiation and accelerates mineralization but also induces death of osteoblasts as they undergo terminal differentiation.

In our experiment, DAG+ cell cultures showed an increasing BMP-2 level in culture media. In contrast, DAG + BMP-2-stimulated MSCs indicate a strong decrease in BMP-2 after the initial peak. In comparison, the unstimulated controls showed a steady decrease of BMP-2 throughout the cultivation period.

One possible explanation for the increasing BMP-2 level under DAG could be an induction of BMP-2 genes by DAG stimulus. It was shown by Liu et al.[48] that DAG induced a 16-fold increase in BMP-6 mRNA in MSCs after 24 h. Controversial to these data, Leclerc et al.[49]

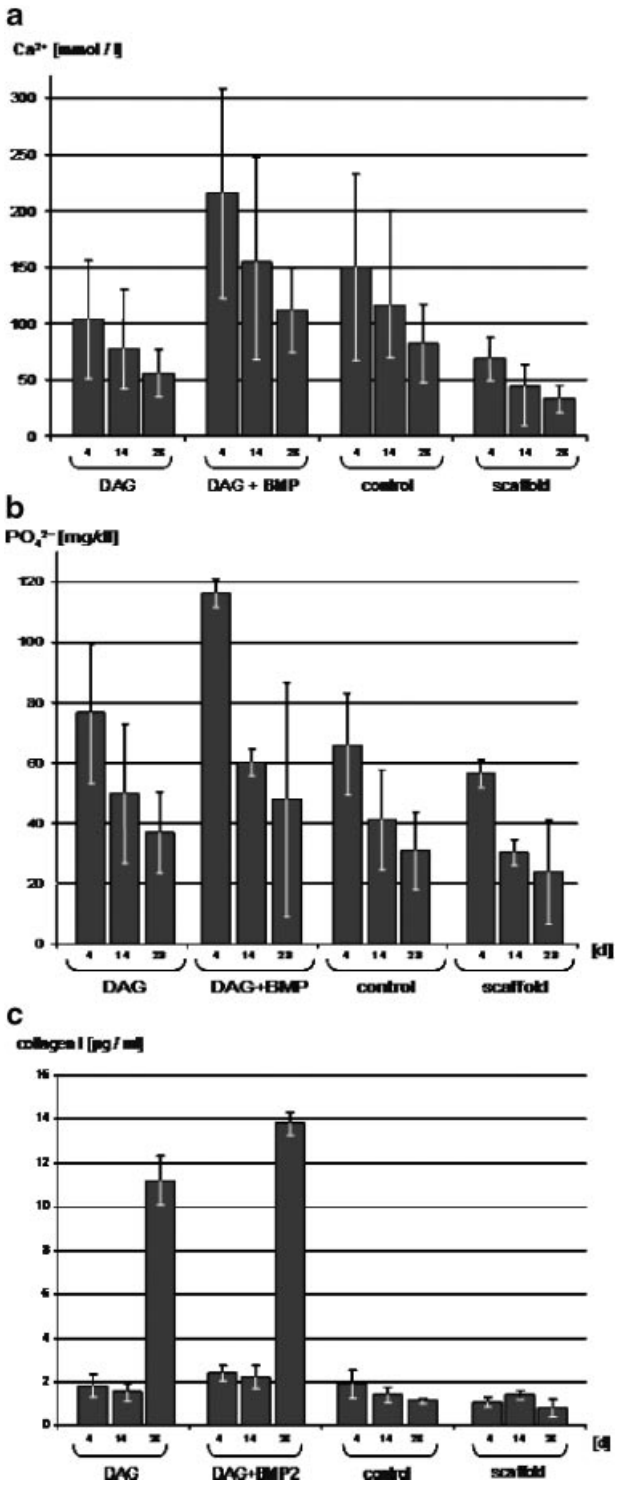

Figure 6. Concentrations of soluble Ca^{2+} (a), PO_4^{2-} (b), and collagen I (c) in culture media at days 4, 14, and 28 in human bone marrow-derived cells, exposed to different stimuli. Both ions showed a steady decrease over cultivation, whereas collagen I shows a peak in cultures which were stimulated by DAG and DAG + BMP-2 at day 28.

reported increased mRNA levels of the BMP antagonists Follistatin and Dan when cells were induced with dexamethasone. Furthermore, dexamethasone was shown to inhibit BMP-2 expression by Luppen et al.[50]

Although some data indicate that the kinase A/C pathways and the Cbfa-1 binding sites of the BMP-2 promotor are involved in transcription regulation of BMP-genes, the exact role of glucocorticoids such as dexamethasone in this pathway remains unclear. There is also evidence that BMP-2 transcription is induced by retinoic acid and also by the phorbol ester phorbol 12-myristate 13-acetate (PMA).[51,52]

The initial peak in BMP-2 level followed by a significant drop for DAG + BMP-2-stimulated cell cultures corresponds to an upregulation of BMP-2 receptors which may able to bind higher amounts of BMP-2. These effects may be superimposed by binding effects of BMP-2 to the fibrous collagen I/III network in DAG + BMP-2/Col-exposed cell cultures.

Yin et al.[53] reported a dose-dependent increase of adipoblast differentiation induced by dexamethasone in marrow stromal cells in vitro. Moreover, 10^{-7} M dexamethasone suppressed the collagen type I gene.

BMP-related effects are complex and signal transduction is mediated by at least three different BMP receptors (BMPR-IA, -IB and -II).[54,55] Several cellular factors suppress a BMP-related differentiation of osteoblasts, such as the nucleocytoplasmic shuttling protein Cas-interacting zinc finger protein (CIZ),[56] tumor necrosis factor (TNF)-alpha, and others.[54]

Our results on human MSCs correspond to Lee et al. who reported a Runx2 expression via Dlx5 induced by BMP-2-stimulated osteoblast-like MC3T3-E1 cells, rat osteosarcoma cell line (ROS17/2.8), and murine bone marrow-derived stromal cells (ST2) in vitro.[57]

The increased level of BMP-2 in culture media of collagen I/III cultures could be also based on BMP-2 retention to the scaffold. Kim and Valentini[58] showed differences in BMP-2 release between hyaluronic acid-based scaffold and collagen gel scaffold which were soak-loaded with rhBMP-2. Here, BMP-2 retention was greater in hyaluronic acid biomaterial than from collagen gels which released most of the initially loaded BMP-2 by 14 days.

Our data show a high interindividual variability of the Ca^{2+} and PO_4^{2-} concentration in culture media of the different groups. In all bone marrow cultures, we found a Ca^{2+} and also a PO_4^{2-} decrease over time. For both molecules, DAG + BMP-2-stimulated cultures showed the highest initial values followed by DAG-stimulated cells. The interactions between Ca^{2+}, PO_4^{2-}, and BMP-2 expression are complex and not understood in detail. It was shown by Honda et al.[59] that increased extracellular calcium levels can stimulate BMP-2 secretion. You have also to consider that osteoblasts can rapidly exchange their intracellular Ca^{2+} storages in response to its differentiation stage and extracellular environment. The closer that the osteoblast is to the mineralization site, the more Ca^{2+} it will possess.[60] Therefore, the decrease of soluble Ca^{2+} and PO_4^{2-} in the culture media can be also interpreted as a sign of progressive biomineralization due to immobilization of these ions within the hydroxapatite lattice.

In future, the application of BMP-2-coated biomaterials is a promising option for the treatment of bony defects as shown for TCP.[61] However, the dosage and kinetics of BMP-2 release is crucial for successful bone regeneration.

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REFERENCES

1. Kanayama M, Hashimoto T, Shigenobu K, et al. 2006. A prospective randomized study of posterolateral lumbar fusion using osteogenic protein-1 (OP-1) versus local autograft with ceramic bone substitute: emphasis of surgical exploration and histologic assessment. Spine 31:1067–1074.
2. Govender S, Csimma C, Genant HK, et al. 2002. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. J Bone Joint Surg [Am] 84-A:2123–2134.
3. Jäger M, Emami R, Thorey F, et al. 2006. Saving implants! BMP-2 application in revision total hip surgery. Int J Biomed Sci 2:187–195.
4. Jaiswal RK, Jaiswal N, Bruder SP, et al. 2000. Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. J Biol Chem 275:9645–9652.
5. Pittenger MF, Mackay AM, Beck SC, et al. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.
6. Mikami Y, Omoteyama K, Kato S, et al. 2007 Inductive effects of dexamethasone on the mineralization and the osteoblastic gene expressions in mature osteoblast-like ROS17/2.8 cells. Biochem Biophys Res Commun 362:368–373.
7. Bruder SP, Jaiswal N, Haynesworth SE. 1997. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem 64:278–294.
8. Tan Y, Wang G, Fan H, et al. 2007. Expression of core binding factor 1 and osteoblastic markers in C2C12 cells induced by calcium phosphate ceramics in vitro. J Biomed Mater Res A 82:152–159.
9. Fu H, Doll B, McNelis T, et al. 2007. Osteoblast differentiation in vitro and in vivo promoted by Osterix. J Biomed Mater Res A 83:770–778.
10. Ishijima M, Tsuji K, Rittling SR, et al. 2007. Osteopontin is required for mechanical stress-dependent signals to bone marrow cells. J Endocrinol 193:235–243.
11. Morelli C, Barbanti-Brodano G, Ciannilli A, et al. 2007. Cell morphology, markers, spreading, and proliferation on orthopaedic biomaterials. An innovative cellular model for the "in vitro" study. J Biomed Mater Res A 83:178–183.
12. Son E, Do H, Joo HM, et al. 2007. Induction of alkaline phosphatase activity by l-ascorbic acid in human osteoblastic cells: a potential role for CK2 and Ikaros. Nutrition 23:745–753.
13. Huang Z, Nelson ER, Smith RL, et al. 2007. The sequential expression profiles of growth factors from osteroprogenitors to osteoblasts in vitro. Tissue Eng 13:2311–2320.
14. Sapkota G, Alarcon C, Spagnoli FM, et al. 2007. Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol Cell 25:441–454.
15. Chan MC, Nguyen PH, Davis BN, et al. A novel regulatory mechanism of the bone morphogenetic protein (BMP) signaling pathway involving the carboxyl-terminal tail domain of BMP type II receptor. Mol Cell Biol 27:5776–5789.
16. Ikeda R, Yoshida K, Inoue I. 2007. Identification of FAZF as a novel BMP2-induced transcription factor during osteoblastic differentiation. J Cell Biochem 101:147–154.
17. Lian JB, Stein GS, Javed A, et al. 2006. Networks and hubs for the transcriptional control of osteoblastogenesis. Rev Endocr Metab Disord 7:1–16.
18. Sapkota G, Knockaert M, Alarcon C, et al. 2006. Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-beta pathways. J Biol Chem 281:40412–40419.
19. Wang Q, Wei X, Zhu T, et al. 2007. Bone morphogenetic protein 2 activates Smad6 gene transcription through bone-specific transcription factor Runx2. J Biol Chem 282:10742–10748.
20. Chen Y, Whetstone HC, Youn A, et al. 2007. Beta-catenin signaling pathway is crucial for bone morphogenetic protein 2 to induce new bone formation. J Biol Chem 282:526–533.
21. Luu HH, Song WX, Luo X, et al. 2007. Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. J Orthop Res 25:665–677.
22. Okamoto M, Dohi Y, Ohgushi H, et al. 2006. Influence of the porosity of hydroxyapatite ceramics on in vitro and in vivo bone formation by cultured rat bone marrow stromal cells. J Mater Sci Mater Med 17:327–336.
23. Domaschke H, Gelinsky M, Burmeister B, et al. 2006. In vitro ossification and remodeling of mineralized collagen I scaffolds. Tissue Eng 12:949–958.
24. Petrovic L, Schlegel AK, Schultze-Mosgau S, et al. 2006. Different substitute biomaterials as potential scaffolds in tissue engineering. Int J Oral Maxillofac Implants 21:225–231.
25. Jäger M, Degistirici O, Knipper A, et al. 2007. Bone healing and migration of cord blood-derived stem cells into a critical size femoral defect after xenotransplantation. J Bone Miner Res 22:1224–1233.
26. Jäger M, Feser T, Denck H, et al. 2005. Proliferation and osteogenic differentiation of mesenchymal stem cells cultured onto three different polymers in vitro. Ann Biomed Eng 33:1319–1332.
27. Jäger M, Fischer J, Schultheis A, et al. 2006. Extensive H(+) release by bone substitutes affects biocompatibility in vitro testing. J Biomed Mater Res A 76:310–322.
28. Bae HW, Zhao L, Kanim LE, et al. 2006. Intervariability and intravariability of bone morphogenetic proteins in commercially available demineralized bone matrix products. Spine 31:1299–1306.
29. Jorgensen NR, Henriksen Z, Sorensen OH, et al. 2004. Dexamethasone, BMP-2, and 1,25-dihydroxyvitamin D enhance a more differentiated osteoblast phenotype: validation of an in vitro model for human bone marrow-derived primary osteoblasts. Steroids 69:219–226.
30. Saito N, Okada T, Horiuchi H, et al. 2003. Local bone formation by injection of recombinant human bone morphogenetic protein-2 contained in polymer carriers. Bone 32:381–386.
31. Xu XL, Lou J, Tang T, et al. 2005. Evaluation of different scaffolds for BMP-2 genetic orthopedic tissue engineering. J Biomed Mater Res B Appl Biomater 75:289–303.
32. Schreiber RE, Blease K, Ambrosio A, et al. 2005. Bone induction by AdBMP-2/collagen implants. J Bone Joint Surg [Am] 87:1059–1068.
33. Oshina H, Sotome S, Yoshii T, et al. 2007. Effects of continuous dexamethasone treatment on differentiation

capabilities of bone marrow-derived mesenchymal cells. Bone 41:575–583.
34. Ito S, Suzuki N, Kato S, et al. 2007. Glucocorticoids induce the differentiation of a mesenchymal progenitor cell line, ROB-C26 into adipocytes and osteoblasts, but fail to induce terminal osteoblast differentiation. Bone 40:84–92.
35. Hong HH, Pischon N, Santana RB, et al. 2004. A role for lysyl oxidase regulation in the control of normal collagen deposition in differentiating osteoblast cultures. J Cell Physiol 200: 53–62.
36. Gerstenfeld LC, Chipman SD, Kelly CM, et al. 1988. Collagen expression, ultrastructural assembly, and mineralization in cultures of chicken embryo osteoblasts. J Cell Biol 106:979–989.
37. Delany AM, Jeffrey JJ, Rydziel S, et al. 1995. Cortisol increases interstitial collagenase expression in osteoblasts by post-transcriptional mechanisms. J Biol Chem 270:26607–26612.
38. Franceschi RT, Iyer BS, Cui Y. 1994. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. J Bone Miner Res 9:843–854.
39. Collin P, Nefussi JR, Wetterwald A, et al. 1992. Expression of collagen, osteocalcin, and bone alkaline phosphatase in a mineralizing rat osteoblastic cell culture. Calcif Tissue Int 50:175–183.
40. Koch H, Jadlowiec JA, Whalen JD, et al. 2005. Osteoblastic differentiation of human adult mesenchymal stem cells after thorough gene transfer of BMP-2 in the absence of dexamethasone. Z Orthop Ihre Grenzgeb 143:684–690.
41. Diefenderfer DL, Osyczka AM, Garino JP, et al. 2003. Regulation of BMP-induced transcription in cultured human bone marrow stromal cells. J Bone Joint Surg [Am] 85-A(Suppl 3):19–28.
42. Diefenderfer DL, Osyczka AM, Reilly GC, et al. 2003. BMP responsiveness in human mesenchymal stem cells. Connect Tissue Res 44(Suppl 1):305–311.
43. Zhou S, Glowacki J, Yates KE. 2004. Comparison of TGF-beta/BMP pathways signaled by demineralized bone powder and BMP-2 in human dermal fibroblasts. J Bone Miner Res 19: 1732–1741.
44. Doss MX, Chen S, Winkler J, et al. 2007. Transcriptomic and phenotypic analysis of murine embryonic stem cell derived BMP2+ lineage cells: an insight into mesodermal patterning. Genome Biol 8:9.
45. Defranco DJ, Lian JB, Glowacki J. 1992. Differential effects of glucocorticoid on recruitment and activity of osteoclasts induced by normal and osteocalcin-deficient bone implanted in rats. Endocrinology 131:114–121.
46. Balint E, Lapointe D, Drissi H, et al. 2003. Phenotype discovery by gene expression profiling: mapping of biological processes linked to BMP-2-mediated osteoblast differentiation. J Cell Biochem 89:401–426.
47. Rai B, Teoh SH, Ho KH, et al. 2004. The effect of rhBMP-2 on canine osteoblasts seeded onto 3D bioactive polycaprolactone scaffolds. Biomaterials 25:5499–5506.
48. Liu Y, Titus L, Barghouthi M, et al. 2004. Glucocorticoid regulation of human BMP-6 transcription. Bone 35:673–681.
49. Leclerc N, Luppen CA, Ho VV, et al. 2004. Gene expression profiling of glucocorticoid-inhibited osteoblasts. J Mol Endocrinol 33:175–193.
50. Luppen CA, Smith E, Spevak L, et al. 2003. Bone morphogenetic protein-2 restores mineralization in glucocorticoid-inhibited MC3T3-E1 osteoblast cultures. J Bone Miner Res 18:1186–1197.
51. Helvering LM, Sharp RL, Ou X, et al. 2000. Regulation of the promoters for the human bone morphogenetic protein 2 and 4 genes. Gene 25:123–138.
52. Rogers MB. 1996. Receptor-selective retinoids implicate retinoic acid receptor alpha and gamma in the regulation of bmp-2 and bmp-4 in F9 embryonal carcinoma cells. Cell Growth Differ 7:115–122.
53. Yin L, Li YB, Wang YS. 2006. Dexamethasone-induced adipogenesis in primary marrow stromal cell cultures: mechanism of steroid-induced osteonecrosis. Chin Med J [Engl] 119:581–588.
54. Singhatanadgit W, Salih V, Olsen I. 2006. Bone morphogenetic protein receptors and bone morphogenetic protein signaling are controlled by tumor necrosis factor-alpha in human bone cells. Int J Biochem Cell Biol 38:1794–1807.
55. Singhatanadgit W, Salih V, Olsen I. 2006. Shedding of a soluble form of BMP receptor-IB controls bone cell responses to BMP. Bone 39:1008–1017.
56. Morinobu M, Nakamoto T, Hino K, et al. 2005. The nucleocytoplasmic shuttling protein CIZ reduces adult bone mass by inhibiting bone morphogenetic protein-induced bone formation. J Exp Med 201:961–970.
57. Lee MH, Kim YJ, Kim HJ, et al. 2003. BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. J Biol Chem 278:34387–34394.
58. Kim HD, Valentini RF. 2002. Retention and activity of BMP-2 in hyaluronic acid-based scaffolds in vitro. J Biomed Mater Res 59:573–584.
59. Honda Y, Anada T, Kamakura S, et al. 2006. Elevated extracellular calcium stimulates secretion of bone morphogenetic protein 2 by a macrophage cell line. Biochem Biophys Res Commun 345:1155–1160.
60. Imai K, Neuman MW, Kawase T, et al. 1992. Calcium in osteoblast-enriched bone cells. Bone 13:217–223.
61. Saito A, Suzuki Y, Kitamura M, et al. 2006. Repair of 20-mm long rabbit radial bone defects using BMP-derived peptide combined with an alpha-tricalcium phosphate scaffold. J Biomed Mater Res A 77:700–706.