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REGULAR ARTICLE

Bone morphogenetic proteins 4 and 2/7 induce osteogenic differentiation of mouse skin derived fibroblast and dermal papilla cells

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Abstract Heterotopic ossification is a pathological condition in which bone forms outside the skeletal system. It can also occur in skin, which is the case in some genetic disorders. In addition to precursor cells and the appropriate tissue environment, heterotopic ossification requires inductive signals such as bone morphogenetic proteins (BMP). BMPs are growth and differentiation factors that have the ability to induce cartilage and bone formation in ectopic sites. The objective of this study is to explore the effect of the BMP-4 homodimer and BMP-2/7 heterodimer on the osteogenic differentiation of primary mouse skin fibroblasts and hair follicle dermal papilla (DP) cells. Osteogenic differentiation was induced by osteogenic induction medium (OS) containing 10 nM dexamethasone. The effect of BMP-4 and BMP-2/7 was studied using alkaline phosphatase (ALP) and calcium assays after 1.5, 3 and 5 weeks of differentiation. Fibroblasts and DP cells were able to differentiate into osteoblast-like matrix mineralizing cells. The first visible sign of differentiation was the change of morphology from rounded to more spindle-shaped cells. BMP-4 and BMP-2/7 exposure elevated ALP activity and calcium production significantly more than OS alone. The osteogenic response to BMP-4 and BMP-2/7 was similar in fibroblasts, whereas, in DP cells, BMP-2/7 was more potent than BMP-4. OS alone could not induce osteogenic differentiation in DP cells. Clear and consistent results show that dermal fibroblasts and stem cells from the dermal papilla were capable of osteogenic differentiation. The BMP-2/7 heterodimer was significantly more effective on hair follicular dermal stem cell differentiation.

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Abbreviations

ALP	alkaline phosphatase
ANOVA	analysis of variance
BM	basic medium
BMP	bone morphogenetic protein
DNAse	deoxyribonuclease
DP	dermal papilla
GNAS	G-protein α-stimulatory subunit gene
HCl	hydrochloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MSC	mesenchymal stem or stromal cells
NaCl	sodium chloride
NaOH	sodium hydroxide
OS	osteogenic medium
PBS	phosphate buffered saline
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
αMEM	minimum essential medium α modification

Introduction

Bone formation is a long-term process that starts during embryonic development when bone is formed by endochondral or intramembraneous ossification. After birth, bone formation is normally restricted to the skeletal system but in pathological conditions such as heterotopic or ectopic ossification, bone is formed outside the skeletal system. Heterotopic ossification can be genetic, post-traumatic, post-surgical, neurogenic, or related to neoplasms or degenerative conditions. It can occur in skin, subcutaneous tissues, skeletal muscle, ligaments and fibrous

tissue next to joints or walls of blood vessels [reviewed in (McCarthy and Sundaram 2005)]. Primary heterotopic ossification in skin is found in several disorders, some of which have a known genetic mutation (Shore and Kaplan 2010). Multiple miliary osteoma cutis (MMOC) is a rare skin disease where tiny nodular osteomas in dermis and nearby subcutaneous tissue show intramembranous ossification. We have studied the etiology of MMOC and found it to be different from G-protein α-stimulatory subunit gene (*GNAS*)-related disorders, which also contain heterotopic intramembranous ossification (Myllyla et al. 2011).

The pathogenesis of heterotopic bone requires precursor cells capable of osteogenic differentiation, the appropriate tissue environment and an inductive signal [reviewed in (Shore and Kaplan 2010)]. In addition to bone marrow mesenchymal stem or stromal cells (MSC) also MSC-like populations from other sources such as adipose tissue (Zuk et al. 2002), umbilical cord blood (Lee et al. 2004) and dental tissues (Huang et al. 2009) have been shown to be able to differentiate into bone forming cells. In skin, dermal fibroblasts (Junker et al. 2010) and hair follicle dermal papilla (DP) cells (Jahoda et al. 2003) also exhibit the potential for osteogenic differentiation. The stromal cells in dermis with fibroblast morphology and potentiality for osteogenic differentiation are in this study, as in many other studies, called fibroblasts (Toma et al. 2005; Lysy et al. 2007; Lorenz et al. 2008), while some studies use broader stromal cell or even mesenchymal stem cell–names (Al-Nbaheen et al. 2013). It should be noted that the exact composition of cells remains largely unknown and primary cell cultures always contain a mixture of different cell types of various differentiation capacity and origin, also in the skin.

Ossification is a complex process, which is regulated by different systemic and local factors such as hormones, growth factors and cytokines (Chau et al. 2009). Bone morphogenetic proteins (BMP) are growth and differentiation factors belonging to the transforming growth factor β superfamily (Wozney et al. 1988). BMPs, 20 of which have been identified so far, are shown to be important in the development and morphogenesis of several tissues and organs (Bragdon et al. 2011). They also have the ability to induce cartilage and bone formation in ectopic sites (Hogan 1996) and some BMPs are used clinically to promote bone healing (Lissenberg-Thunnissen et al. 2011). Human intramembranous and endochondral bones show different patterns of BMP expression. Various studies suggest that BMP-4 and BMP-7 are more strongly related to intramembranous ossification (Aono et al. 1995; Suttapreyasri et al. 2006). The environment affects the type of bone formation; fibroblasts transduced with the BMP-2 gene induced endochondral ossification after intramuscular injection and intramembranous ossification when put into calvarial defects (Wang et al. 2009).

Individual BMPs can induce bone formation but there is evidence that they normally work together. For example, BMPs 2, 4 and 7 are expressed in overlapping patterns during limb development (Lyons et al. 1995; Nishimatsu and Thomsen 1998) and fracture healing (Cho et al. 2002). BMP heterodimers are reported to have greater biological activity than their constituent homodimers (Aono et al. 1995; Zhao et al. 2005; Zheng et al. 2010). BMPs are also expressed in skin. Hee and Nicoll (Hee and Nicoll 2009) showed that BMP signaling plays a role in osteoblastic differentiation of dermal fibroblasts stimulated by vitamin D. Plikus et al. found that periodic expression of BMP-2 and BMP-4 in mouse dermis regulates hair regeneration (Plikus et al. 2008). Our previous findings suggest that BMP-2 and BMP-4 are present in epidermal keratinocytes, dermal fibroblasts and endothelial cells and in skin adnexa (Myllyla et al. 2011).

It has been previously shown that fibroblasts and DP cells are able to differentiate toward an osteogenic lineage (Junker et al. 2010, Jahoda et al. 2003). However, the effect of BMPs on osteogenic differentiation has been studied mostly on mesenchymal stem or stromal cells and preosteoblasts (MC3T3 cells) (Zhao et al. 2005, Kang et al. 2009, Zheng et al. 2010). The present work is an attempt to investigate the osteogenic differentiation of skin derived mesenchymal cells as part of the skin ossification process. As dermis contains cells capable of osteogenic differentiation, we tested their putative inducers for mineralization. We have previously shown that ossification in MMOC is direct or intramembranous in nature and here we analyzed whether the BMP-4 homodimer and BMP-2/7 heterodimer, which are suggested to be involved in direct ossification, affect the alkaline phosphatase activity and mineralization capability of mouse skin fibroblasts and DP cells.

Materials and methods

Skin fibroblast isolation and culture

Nine- to 10-week-old FVB/N HanHsd male mice were sacrificed at the Laboratory Animal Centre of the University of Oulu. The fibroblast isolation method was modified from Seluanov et al. (Seluanov et al. 2010). Briefly, hair from the breast area was removed by an electric shaver and the skin was rapidly cleaned with 70 % ethanol. The skin from the breast (area between front paws) was handled with forceps and scissors to carefully separate the skin from underlaying tissue to expose the dermal side of the skin and to avoid any contamination with keratinocytes. Small pieces of dermis only and underlying fascia were cut under a stereomicroscope and placed in a petri dish containing Ca_2/Mg_2 free phosphate buffered saline (PBS). The tissue pieces were cleaned of adipose tissue and parts containing hair were discarded. The

tissue pieces were moved to a new petri dish containing αMEM, 500 U/ml collagenase I (Worthington) and 20 U/ml DNAse I. The tissue pieces were cut to 1 × 1mm pieces using scalpels. Digestion was carried out for 30–90 minutes at +37 °C with occasional suspensions until the tissue was broken into barely visible pieces. One volume of cell culture medium containing αMEM, 13.5 % heat-inactivated fetal bovine serum (HyClone), 2 mM L-glutamine, 100U/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES and 1.25 μg/ml amphotericin B (Invitrogen) was added and the suspension was centrifuged at 190 g for 5 minutes. The cells were suspended in 10 ml cell culture medium, centrifuged again and plated in 5 ml culture medium in a 25 cm^2 cell culture flask. The cells were cultured at +37 °C in a humidified atmosphere containing 5 % CO^2. The medium was changed every 3–4 days. The amount of serum was decreased to 10 % after the first division. The cells were divided when the culture was 70-80 % confluent and used for experiments in the 3rd passage.

All materials were from Sigma–Aldrich (Dorset, UK) if not otherwise stated. All animal experimentation was approved by the Animal Care and Use Committee of the University of Oulu.

Hair follicle dermal papilla (DP) cell isolation and culture

Seven- to 9-week-old FVB/N HanHsd male mice were sacrificed at the Laboratory Animal Centre of the University of Oulu. Hair follicle dermal papilla (DP) cells were isolated as previously described (Jahoda et al. 1991). Papillas were plated in separate wells in 4-well plates, up to 15 per well and cultured in media containing αMEM, 20 % heat-inactivated fetal bovine serum (HyClone), 2 mM L-glutamine, 0.5 mg/ml gentamycin and 2.5 μg/ml amphotericin B (Invitrogen). The amount of serum was decreased to 10 % after the first division. The cells were divided when the culture was 70-80 % confluent, and used for experiments in the 3rd passage.

Osteogenic differentiation

Cells were cultured in 24-well plates for 10, 21, or 35 days in four different media. Osteogenic induction medium, containing 10 nM dexamethasone, 10 mM β-glycerophosphate (Fluka Biochemica) and 0.17 mM ascorbic acid-2-phosphate in addition to basic cell culture medium, was used for the first 7 days. BMP-4 homodimer or BMP-2/7 heterodimer (R&D Systems) was added to a final concentration of 50 ng/ml to the osteogenic induction medium. After the first week, the osteogenic induction medium was replaced by an osteogenic medium containing β-glycerophosphate and ascorbic acid-2-phosphate but not dexamethasone or BMPs, because there is some evidence that glucocorticoids can inhibit proliferation of osteoblasts and production of bone in later phases of bone formation (Walsh et al. 2001). The basic cell culture medium, containing αMEM, 10 % heat-inactivated special fetal bovine serum (Gibco), 2 mM L-glutamine, 100U/ml penicillin, 0.1 mg/ml streptomycin and 10 mM HEPES, was used as a control. Cells were plated at 5, 000 per well in five replicate wells. For ALP and von Kossa staining the cells were cultured on coverslips.

Alkaline phosphatase (ALP) assay

Alkaline phosphatase activity was measured after 10 days of osteogenic differentiation. For ALP histochemical staining, the cells were washed with PBS, fixed with citrate-acetone formaldehyde fixative, washed with deionized water and stained for enzyme activity with alkaline solution containing naphtol AS-BI phosphate and fast red violet LB base according to the manufacturer's instructions (alkaline phosphatase kit, Sigma–Aldrich). For ALP quantitative activity, the cells were washed with PBS, the lysis buffer containing 50 mM Tris–HCl, 0.1 % Triton-X-100 and 0.9 % NaCl, pH 7.6 was added to the cells and the plates were frozen at least overnight at −70 °C to complete the lysis. After thawing and mixing the content of the wells, ALP enzyme activity was determined by using 0.1 mM 4-*p*-nitrophenylphosphate as a substrate in an assay buffer containing 0.1 M Tris and 1 mM $MgCl_2$, pH 10. After the reaction mixture had been incubated for 30 minutes at room temperature, the reaction was stopped by the addition of 1.0 M NaOH and the absorbance was read at 405 nm in a plate reader (Victor 2, Wallac Oy, Turku, Finland). Five parallel samples were measured in duplicate (in DP cells only, 2–3 parallel samples). The protein contents of the wells were determined by a Bio-Rad Protein Assay (Bio-Rad Laboratories) with bovine serum albumin as the standard. The specific ALP activity was expressed as absorbance at 405 nm / protein mg/ml.

Calcium analysis

The calcium content was determined after 21 or 35 days of osteogenic differentiation. For von Kossa silver staining of mineralized nodules, the cells were washed with PBS, covered with 1% silver nitrate ($AgNO_3$) for 30 minutes under UV light, washed with deionized water, covered with 2.5% sodium tiosulphate for 5 minutes and washed with deionized water. For quantitative calcium concentration analysis, the cells were washed with PBS, 0.6 M HCl was added to the cells and they were incubated overnight at room temperature. The calcium content was determined based on the reaction of calcium with o-cresolphthalein-complexone according to the manufacturer's instructions (Roche Diagnostics Corporation, Mannheim, Germany). The colorimetric reaction was measured at 540 nm in a plate reader (Victor 2, Wallac Oy, Turku,

Finland). Five parallel samples were measured in duplicate (in DP cells only 2–3 parallel samples).

Statistical analysis

Statistical analyses were performed by an IBM Statistical Package for the Social Sciences (IBM SPSS; version 19, Chicago, IL, USA) using one-way ANOVA and Scheffe and Tukey HSD PostHoc tests. The normality of distributions was tested by the Kolmogorow–Smirnov test. Values of $P<0.05$ were considered as significant. Graphs are drawn with Origin Pro 8.6.0 (OriginLab Corp., Northampton, MA, USA). Figures are created with Paint Shop Pro (Jasc Software, Eden Prairie, MN, USA).

Results

Skin fibroblasts

Mouse skin fibroblasts were able to differentiate into calcium-producing ALP-positive cells. The first visible sign of differentiation was the morphology change from round to more spindle-shaped cells (Fig. 1). BMP-4 and BMP-2/7 elevated ALP activity (Fig. 2) and calcium concentration (Fig. 3) significantly more than osteogenic induction medium alone. The effect of BMP-4 and BMP-2/7 was similar in magnitude in comparison to OS alone. It is notable that the BMP addition effect on mineral production was evident at 3 weeks (Fig. 3) and at 5 weeks there was no difference in deposited calcium between the OS and OS+BMP groups. The results were confirmed by three independent experiments with cells isolated from three different mice (two experiments in the 5 weeks calcification assay).

Hair follicle dermal papilla (DP) cells

In order to study the specificity of the BMP response in skin cells, we used isolated hair follicle dermal papilla (DP) cells for similar osteoinduction studies. These results suggest that osteogenic induction medium alone could not induce osteogenic differentiation in these cells (Fig. 4). However, osteogenic induction medium containing BMP-4 or BMP-2/7 could induce DP cells to differentiate into calcium-producing cells. In these cells, BMP-2/7 seemed to be more potent than BMP-4. The results were confirmed by two independent experiments with cells isolated from four mice (cells from three mice combined to obtain sufficient cells).

Discussion

Heterotopic ossification in skin is found in several disorders. A mutation in one of the receptors for BMPs causes endochondral

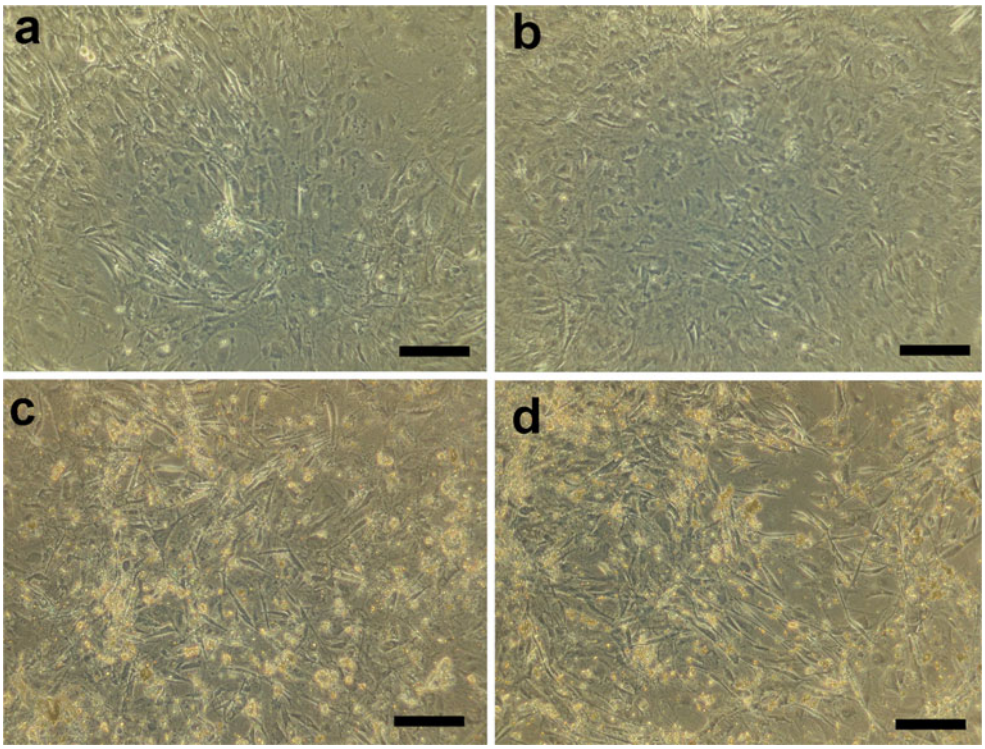

Fig. 1 Skin fibroblasts after two weeks osteogenic differentiation. The cells cultured in basic medium (**a**) or osteogenic medium (**b**) are round and no calcium deposits can be seen. Both BMP-4 (**c**) and BMP-2/7 (**d**) treated cells are more spindle-shaped and calcium deposits can be clearly seen. Pictures are taken by phase contrast microscope. *BMP*=bone morphogenetic protein. *Scale bar* 200 μm

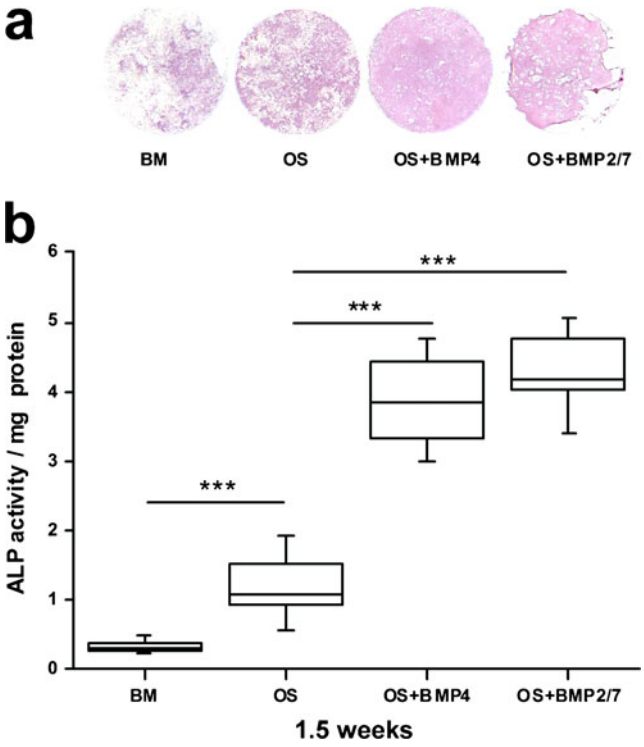

Fig. 2 Alkaline phosphatase (ALP) activity in fibroblasts after 1.5 weeks osteogenic differentiation. **a** Histochemical staining for ALP activity, representative pictures are shown. **b** In pNPP-based assay, the activity is presented in relation to the amount of total protein. Osteogenic medium (OS) alone could elevate alkaline phosphatase activity compared to basic medium (BM). Both BMP-4 and BMP2/7 could induce significantly more ALP activity than BM or OS alone. *ALP* = alkaline phosphatase, *BM* = basic medium, *OS* = osteogenic medium, *BMP* = bone morphogenetic protein, pNPP = 4-p-nitrophenylphosphate. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$

heterotopic ossification in fibrodysplasia ossificans progressiva and a mutation in the *GNAS*-gene is linked to intramembranous ossification in skin in several syndromes such as progressive osseous heteroplasia and Albright hereditary osteodystrophy (Shore and Kaplan 2010). In MMOC, the bone nodules in skin also show intramembranous ossification but the etiology seems to be different from *GNAS*-gene-based disorders. The origin of the osteoma-forming cells in MMOC is not known (Myllyla et al. 2011), although it is known that in skin there are several mesenchymal cell types, e.g., dermal fibroblasts (Junker et al. 2010) and hair follicle dermal papilla (DP) cells (Jahoda et al. 2003) that can potentially undergo osteogenic differentiation. The definition of osteogenic differentiation is rather difficult. Usually, functional tests such as alkaline phosphatase (ALP) activity and calcium deposition are used (Prince et al. 2001).

Our results show that both mouse skin fibroblasts and DP cells are able to undergo osteogenic differentiation. In fibroblasts, the dexamethasone containing osteogenic medium alone is sufficient to induce osteogenic differentiation, as shown by elevated ALP activity and calcium production. In contrast, in DP cells osteogenic medium alone could not induce osteogenic differentiation. Previously, Jahoda et al. have shown that DP cells can differentiate into calcium producing cells, although only one fourth of their clonal dermal papilla cell lines produced calcium (Jahoda et al. 2003). They discussed that in hair follicle dermal papilla, there might be a mixture of committed progenitor cells from different lineages, which could explain this difference in results. Another possibility is that there are few cells with broader capabilities and different mixtures of signaling factors push these cells to different lineages.

In addition to precursor cells capable of osteogenic differentiation and the appropriate micromilieu, heterotopic ossification also requires an inductive signal (Shore and Kaplan 2010), such as BMPs, which also have the ability to induce cartilage and bone formation in ectopic sites (Wozney et al. 1988). Among plenty of BMPs, BMP-4 and BMP-2/7 were chosen for this study, while BMP-4 and BMP-2 are present in skin (Myllyla et al. 2011; Plikus et al. 2008) and BMP-4 and BMP-7 more strongly related to intramembranous than endochondral ossification (Aono et al. 1995; Suttapreyasri et al. 2006). As summarized in Table 1, all BMPs had a significant effect on osteogenic differentiation of both dermal fibroblasts and DP cells. There was no significant difference in the osteogenic potential of the BMP-4 homodimer and BMP-2/7 heterodimer in fibroblasts. This is interesting, since combined transduction with adenoviruses containing BMP-7 and BMP-2 or BMP-4 has been shown to have more potent osteogenic induction capacity than transduction of individual adenoviruses containing BMP-2, -4, or −7 alone on several mesenchymal cell lines (Zhao et al. 2005). It has also been shown that the BMP-2/7 heterodimer has lower threshold concentrations but similar maximum effects in inducing osteoblastogenesis of the preosteoblast cell line (MC3T3-E1) compared with BMP-2 and BMP-7 homodimers (Zheng et al. 2010).

The change in cell morphology to more spindle-shaped after BMP treatment might be due to sensitivity of our cells to BMP-induced remodeling in actin cytoskeleton and focal adhesions, which regulates fibroblast migration without affecting cell proliferation (Konstantinidis et al. 2011). BMP-2 has been shown to affect the motility of tumor cells in many cancers, e.g., in gastric cancer (Kang et al. 2010, Deng et al. 2007) and BMP-4 increased migration and invasion in a colon cancer study, where BMP-4 treated cells showed spindle-shaped invasive phenotype (Deng et al. 2007). These BMP-induced changes in cell morphology are interesting and not fully characterized here and the subject needs further investigation.

In contrast to fibroblasts, DP cells were able to differentiate into mineralizing cells only when BMP-4 or BMP-2/7 was added to the osteogenic medium for 1 week. Rendl et al. have shown that BMP-signaling is important for DP cells to maintain their key characteristic features in vitro (Rendl et al. 2008). The BMP-2/7 heterodimer was significantly more

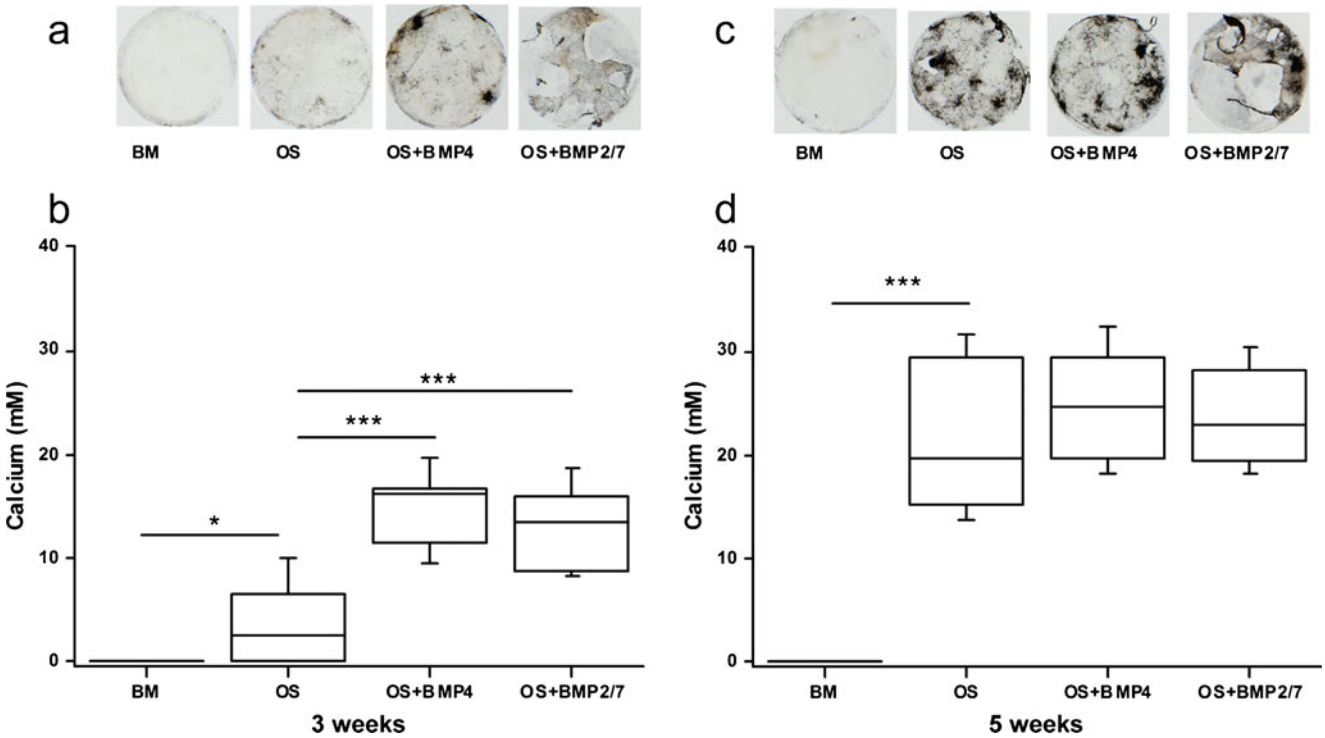

Fig. 3 Calcium production in fibroblasts after 3 (**a**-**b**) and 5 weeks (**c**-**d**) osteogenic differentiation. Mineralized nodule formation is illustrated by von Kossa staining (**a** and **c**), representative pictures are shown. The quantitative calcium content was determined based on the reaction of calcium with o-cresolphthalein-complexone (**b** and **d**). Osteogenic medium (OS) alone could elevate calcium concentration slightly after 3 weeks (**b**) and more significantly after 5 weeks (**d**) differentiation compared to basic medium (BM). Both BMP-4 and BMP-2/7 could induce significantly more calcium production than OS alone after 3 weeks differentiation. There were no significant differences between the levels induced by BMP-4 and BMP-2/7. *BM* = basic medium, *OS* = osteogenic medium, *BMP* = bone morphogenetic protein. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$

potent than the BMP-4 homodimer for inducing calcium production of DP cells. This is interesting, since the BMP-4 homodimer and BMP-2/7 heterodimer were equally potent in fibroblasts. The reason might be that BMP-signaling is especially important in the hair follicle area and is therefore probably very well-regulated and the type of BMP is significant. This might also explain why the osteogenic medium alone could not induce osteogenic differentiation in DP cells. BMP signaling may not be so carefully regulated around dermal fibroblasts, which react also on pure osteogenic medium. Therefore, the type of BMPs may not be as important in their differentiation as it seems to be in DP cells.

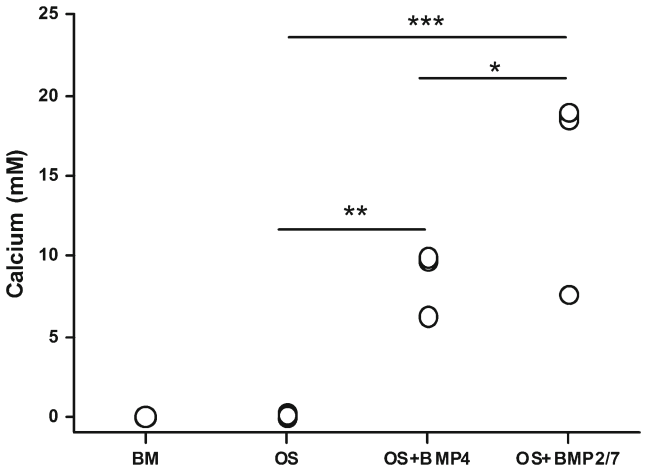

Fig. 4 Calcium concentration in dermal papilla cells after 3 weeks osteogenic differentiation. Osteogenic medium (OS) alone could not elevate the calcium concentration. Both BMP-4 and BMP-2/7 could induce significantly more calcium production than BM or OS alone. BMP-2/7 could induce significantly more calcium production than BMP-4. *BM* = basic medium, *OS* = osteogenic medium, *BMP* = bone morphogenetic protein. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$

Table 1 Summary of osteoinduction data. ALP and calcium deposition results are summarized as an osteoinduction score for the different treatments and cell lines used

	Skin fibroblasts	Dermal papilla cells
OS	+	no effect
OS+BMP-4	+++	++
OS+BMP-2/7	+++	+++

+++ = strong effect, ++ = intermediate effect, + = mild or delayed effect in all performed assays

When BMPs are used clinically for induction of bone healing, only one recombinant BMP is usually used (Lissenberg-Thunnissen et al. 2011). Our data indicate that this could be enhanced by use of BMP-2/7 or even BMP-4 in combination, as already shown earlier for a different combination of BMPs (Wutzl et al. 2010). A limitation in this study was the use of mouse cells. Further work is needed to compare these results to human cell cultures, since the effects of BMPs on rodent cells may differ from the effects on human cells (Osyczka et al. 2004).

To conclude, our clear and consistent results demonstrate that primary mouse skin fibroblasts and hair follicle dermal papilla cells are both able to differentiate into osteoblast-like matrix mineralizing cells. Both the BMP-4 homodimer and BMP-2/7 heterodimer had significant effects on the osteogenic differentiation of these cells and for the DP cells BMP exposure is essential. The results of this study demonstrate that in skin there are precursor cells capable of osteogenic differentiation, which could explain the pathogenesis of MMOC and other heterotopic ossifications. These cells and signaling factors, such as BMPs, can induce a matrix mineralization process in the appropriate micromilieu and later mineralization itself serves as a new mineralization center for further bone formation.

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