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RESEARCH ARTICLE

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Osteoinduction within adipose tissue fragments by heterodimeric bone morphogenetic Proteins-2/6 and -2/7 versus homodimeric bone morphogenetic protein-2: Therapeutic implications for bone regeneration

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Abstract

Background: Fragments of subcutaneous adipose tissue that have been genetically modified to express bone morphogenetic protein-2 (BMP-2) regenerate large segmental osseous lesions in rodents. Gene-activated adipose tissue can be implanted into osseous defects without prior cell extraction and cell culture. The present study aimed to explore whether the heterodimers BMP-2/6 or BMP-2/7 exceed the osteoinductive effect of BMP-2 on adipose tissue.

Methods: In an *in vitro* tissue culture system, freshly harvested rat subcutaneous adipose tissue was cultivated in the presence of either BMP-2 or BMP-2/6 or BMP-2/7 at a high (200 ng/ml) and low (50 ng/ml) concentration. Gene expression analysis as well as histological and immunohistochemical methods were applied to test for osteoinduction.

Results: A concentration of 200 ng/ml of homodimeric BMP-2 induced osteogenic differentiation most potently, showing more calcification and a higher expression level of bone markers than both concentrations of BMP-2/6 or -2/7. A concentration of 50 ng/ml of BMP-2 was a significantly stronger osteogenic inducer than both concentrations of BMP-2/6 and the low concentration of BMP-2/7. The most potent heterodimeric driver of osteoinduction was BMP-2/7 at a high concentration, demonstrating effects similar to those of BMP-2 at a low concentration.

Conclusions: Homodimeric BMP-2 evoked osteoinduction within adipose tissue more potently and at a lower concentration than heterodimeric BMP-2/6 or BMP-2/7. This result agrees well with the fact that it might be easier to translate adipose grafts activated by homodimeric BMP-2 clinically. Preclinical *in vivo* gene transfer studies are necessary to confirm the results of the present study.

KEYWORDS
adipose tissue, BMPs, bone healing, gene transfer, heterodimers, osteoinduction

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1 | INTRODUCTION

Scientists continue to search for novel clinically applicable approaches allowing the repair of bone both reliably and cost-effectively.[1] The current standard procedure employed in clinics to treat bony non-unions and osseous defects is transplantation of autologous bone. Unfortunately, this method is not always successful and has several drawbacks.[2] Hence, it is very important to discover and develop molecular therapeutic approaches to induce growth of new bone.

Bone morphogenetic proteins (BMPs) belong to the most widely studied molecules inducing osteogenic differentiation and proliferation of progenitor cells. In 2002, the US Food and Drug Administration approved BMP-2. Following approval, BMP-2 was applied to treat bony non-unions[3] and fractures,[4] for spine fusion,[5] and to repair bone in the jaw.[6] However, delivery of human recombinant BMP-2 requires improvement because it has a short biological half-life.[7] This is where gene therapy approaches came into play, allowing application of reduced quantities of bone growth-stimulating molecules at the site of injury for several weeks.[8]

BMP-2 transduced adipose-derived stem cells differentiate into bone cells *in vitro* and can heal bony lesions *in vivo*.[9,10] Despite their preclinical success, such traditional cell-based gene transfer strategies possess certain drawbacks preventing their widespread clinical application: they are time-consuming and expensive as a result of the need for the isolation and expansion of cells.[11] Also, applying conventional *ex vivo* gene therapy means that two invasive treatments are performed for cell harvest and cell re-implantation.[12] For these reasons, several research groups are working on expedited gene therapy approaches that can be translated from bench to bedside.[1,12,13]

The use of adipose tissue grafts genetically modified to secrete growth factors avoids the extraction of cells and cell culture and has the potential for a single-surgery approach.[12] It was reported that BMP-2-transduced subcutaneous adipose tissue fragments reliably heal segmental bone defects of a critical size in rodents.[14] In another study, implantation of BMP-7 gene-activated adipose tissue grafts into the same rat segmental bone defects led to bone repair as well.[15] However, modification with BMP-2 cDNA induced a much more intense, reliable and robust healing response compared to BMP-7. In both cases, the gene-activated adipose tissue fragments served as natural three-dimensional, biodegradable, osteo-regenerative implants representing a source of progenitor cells and growth factor molecules.[12]

Several studies have demonstrated that it is possible to achieve improved healing of bone by gene therapy using combinations of growth factors.[16–18] Moreover, scientists have successfully employed BMP heterodimers *in vitro* and *in vivo* and found that osteoinduction can be elicited more effectively by heterodimers than with the individual homodimers.[19,20] At present, it is unknown whether heterodimeric BMPs can further enhance osteoinduction within adipose tissue. Therefore, the present study aimed to compare the osteoinductive effect of heterodimeric BMP-2/6 and BMP-2/7 on adipose tissue fragments versus the homodimer BMP-2. The *in vitro* adipose tissue culture system utilized in the present study has been applied previously.[21,22] It has been shown that, under the influence of BMPs, fragments of subcutaneous adipose tissue possess the capability of transdifferentiation into tissue with bone-like characteristics to a certain degree.[23] This basic science study may help to make a decision regarding the design of future expedited *ex vivo* gene therapy investigations selecting the most promising growth factor gene.

2 | MATERIALS AND METHODS

2.1 | Design of the experiment

Two male Fischer rats (F-344/DuCrl) served as donors for subcutaneous adipose tissue. Complete fresh fragments of adipose tissue were randomly distributed to eight experimental groups. Adipose tissue fragments were cultured under various conditions: control groups in normal growth medium or osteogenic medium alone, experimental groups in osteogenic medium with the addition of 50 ng/ml or 200 ng/ml of recombinant human BMP-2 or recombinant human BMP-2/6 or recombinant human BMP-2/7 (R&D Systems, Minneapolis, MN, USA; Bio-Techne Ltd, Abingdon, UK) for up to 20 days. Bone-related markers on the gene and protein level, as well as calcium deposition, were measured to test for osteoinduction within adipose tissue. The present study was carried out in accordance with the guidelines of the university's and government's responsible committees.

2.2 | Harvest of adipose tissue

After sacrificing Fischer 344 rats by isoflurane (Abbott GmbH & Co. KG, Ludwigshafen, Germany), subcutaneous adipose tissue was excised and rinsed in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (Biochrom Ltd, Cambourne, UK) supplemented with 180 IU/ml penicillin/streptomycin and 0.375 μg/ml amphotericin (Biochrom Ltd). Then, adipose tissue fragments of a standardized size (4 mm diameter, approximately 1 mm thickness) were created.

2.3 | Culture of adipose tissue

Adipose tissue was cultivated at 37° C and 5% CO_2, whereas change of medium taking place every second day. Cells were not isolated. Instead, complete adipose tissue fragments were held in culture for up to 20 days. Four pieces of adipose tissue were placed in one well of a 24-well plate (Nunclon; Thermo Scientific, Waltham, MA, USA). A comparison of eight groups/culture conditions was performed: (i) group NM: adipose tissue with normal growth medium [DMEM/Ham's F-12, 10% fetal calf serum (FCS)]; (ii) group OM: adipose tissue with osteogenic medium (DMEM/Ham's F-12, 10% FCS, 10 nM dexamethasone, 10 mM β-glycerophosphate, 50 μM L-ascorbic acid 2-phosphate); (iii) groups 3–8 were cultured in osteogenic medium supplemented with either 50 ng/ml or 200 ng/ml of rhBMP-2,

rhBMP-2/6 or rhBMP-2/7. Adipose tissue samples were investigated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), histology and immunohistochemistry on days 10 and 20.

2.4 | Analysis of expression of bone marker genes by qRT-PCR

Bone sialoprotein (*BSP*), *Alkaline phosphatase* (*ALP*), *Collagen 1a1 (Col1a1)*, *Runt-related transcription factor 2* (*RUNX-2*), *Osteocalcin* (*OCN*) and *Osteopontin* (*OPN)* were assayed by qRT-PCR. Primers were purchased from Qiagen (Valencia, CA, USA). Total mRNA was extracted from adipose tissue. All measurements were carried out in triplicate. Liquid nitrogen was used to freeze adipose tissue and homogenization was performed to prepare for total mRNA extraction. QIAzol Lysis Reagent (Qiagen) was applied in accordance with the manufacturer's recommedations. The concentration and purity of total RNA were determined at $A_{260/280}$. Reverse transcription was then performed with a QuantiTect Reverse Transcription-Kit (Qiagen). qRT-PCR was carried out using a FastStart Essential DNA Green Master (Roche Diagnostics, Basel, Switzerland) and a Light Cycler 96 (Roche Diagnostics). Calculation of relative gene expressions was performed with the $2^{-\Delta\Delta Ct}$ method using the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*).

2.5 | Histology and detection of calcium

For histological evaluation, six adipose tissue samples from each group were utilized on days 10 and 20 of tissue culture. Following fixation in 4% formalin, samples were dehydrated and embedded in paraffin using a tissue processor STP-120 (Thomas-Medical, Indianapolis, IN, USA). After embedding of the adipose tissue fragments in paraffin blocks, a RM2255 microtome (Leica Microsystems, Wetzlar, Germany) was used to cut 8-μm sections. Then, mounting of sections on superfrost glass slides (Menzel; Thermo Scientific), deparaffinization and rehydration followed. A freshly prepared 40 mM Alizarin red S solution (Sigma, St Louis, MO, USA) with a pH of 4.1 was used for staining and detection of calcium. This was followed by rinsing with phosphate-buffered saline (PBS), dehydration in increasing gradients of ethanol and clearing in Rothistol (Roth, Karlsruhe, Germany). After mounting samples with DPX (Merck, Darmstadt, Germany), all specimens were evaluated with a M8 Microscope (PreciPoint, Freising, Germany) and ViewPoint Scanner software (VuPoint Solutions, City of Industry, CA, USA).

2.6 | Immunohistochemistry

Immunohistochemistry was conducted to check the presence of the marker proteins sclerostin (Scl), osteocalcin (OCN) and osteopontin (OPN) within adipose tissue. Sections were washed using buffer (1 × PBS with 0.1% Brij Antigen; Sigma), followed by blocking of endogenous peroxidase activity. A SuperVision 2 HRP single species kit (DCS Innovative GmbH, Hamburg, Germany) was used in accordance with the manufacturer's recommedations and 1:100 diluted primary antibodies Scl and OPN and OCN (rabbit anti-rat) (Biorbyt, Cambridge, UK) were applied. Mayer's hematoxylin was used for counterstaining. Finally, all specimens were photographed and evaluated employing ViewPoint Scanner software (VuPoint Solutions) and the M8 Microscope (PreciPoint).

2.7 | Histomorphometry

Histomorphometric evaluation was performed on six adipose tissue specimens from each group. For the quantification of the percentage of stained area of cross-sections of the adipose tissue samples on days 10 and 20, ImageJ, version 1.6 (NIH, Bethesda, MD, USA) was used. Measurements were performed in triplicate.

2.8 | Statistical analysis

Statistical calculations were performed with Excel 2010 (Microsoft Corp., Redmond, WA, USA) and Prism, version 5.02 (GraphPad Software Inc., San Diego, CA, USA). To determine differences, a paired *t* test for two-group comparisons was used. The Mann–Whitney *U* test was applied when the assumptions of a *t* test were not met. Mean ± SE values are shown. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Analysis of gene expression

In comparison with adipose tissue cultivated with normal medium (NM), samples cultured in osteogenic medium (OM) showed significantly elevated levels of bone-related gene expression, except for RUNX-2 at 10 days (Figure 1B) and COL1A1 at 20 days (Figure 1C). The homodimer BMP-2 induced expression of bone-related genes at 200 ng/ml most potently. A concentration of 200 ng/ml BMP-2 caused higher gene expression of bone markers than both concentrations of the heterodimeric BMP-2/6 or -2/7 at both time points. All bone-related genes showed the highest levels in the BMP-2 200 ng/ml group. In the BMP-2 200 ng/ml group, the early marker ALP was approximately 8.3-fold overexpressed at day 10 and approximately 5.5-fold overexpressed at day 20 (Figure 1A). By contrast, the heterodimers increased the expression of ALP at the high concentration by only approximately 3.6-fold at day 10 and 2.8-fold at day 20 (BMP-2/7 200 ng/ml), and approximately 3.1-fold at day 10 and 2.4-fold at day 20 (BMP-2/6 200 ng/ml), respectively (Figure 1A). A concentration of 50 ng/ml of BMP-2 induced significantly higher expression levels of bone-related genes than both doses of BMP-2/6 and the low dose of BMP-2/7, except for ALP on day 20 and Col1a1

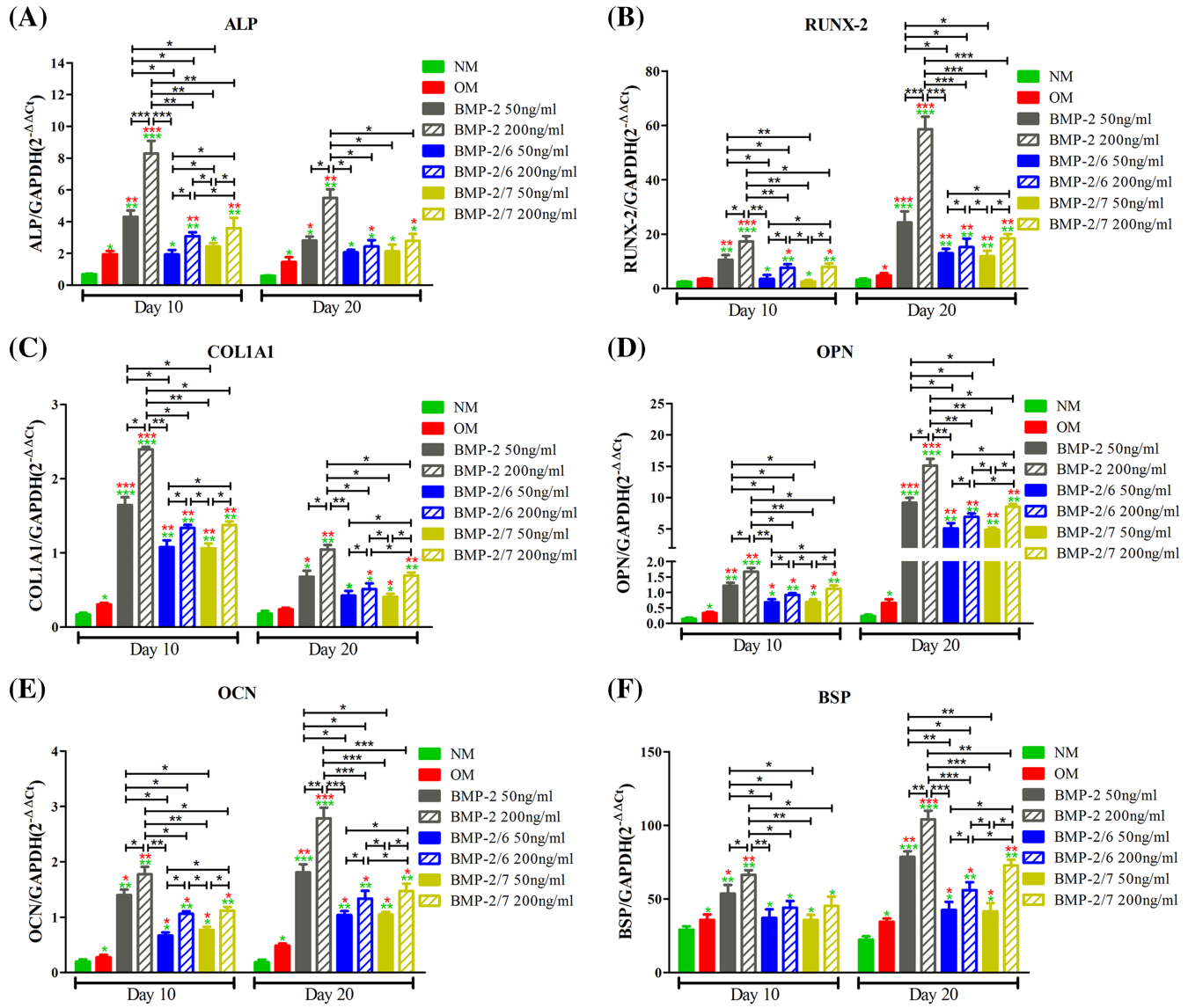

FIGURE 1 Analysis of gene expression by qRT-PCR. Expression of bone-related genes by adipose tissue samples was analyzed by qRT-PCR. Analysis of gene expression was carried out in triplicate. (A) ALP; (B) RUNX-2; (C) Col1a1; (D) OPN; (E) OCN; and (F) BSP; NM, normal medium; OM, osteogenic medium

on day 20, with no significantly difference between these groups. BMP-2/7 200 ng/ml was the most potent heterodimeric inducer of osteoinduction as analyzed on the gene expression level, showing similar effects as BMP-2 50 ng/ml. Expression of RUNX-2 induced by 200 ng/ml of BMP-2 was approximately 2-fold higher compared to 200 ng/ml of BMP-2/7 on day 10, and approximately 3-fold higher compared to 200 ng/ml of BMP-2/7 on day 20 (Figure 1B). An approximately 1.7-fold higher Col1a1 expression was noted on day 10 for the high concentration of BMP-2 compared to the high concentration of BMP-2/7 (Figure 1C). The strongest increase of OCN was induced by BMP-2 200 ng/ml on day 20, which was approximately 2-fold higher than the expression level evoked by BMP-2/7 200 ng/ml (Figure 1E). At both time points, the highest expression levels of OPN (Figure 1D) and BSP (Figure 1F) were noted for BMP-2 200 ng/ml. The ability of 50 ng/ml BMP-2/6 and 50 ng/ml BMP-2/7 to induce bone-related gene expression was not significantly different. Only ALP at day 10 was higher when induced by 50 ng/ml of BMP-2/7 compared to 50 ng/ml of BMP-2/6 (Figure 1A).

3.2 | Alizarin red S staining

No calcium deposition was detected in adipose tissue fragments cultured in normal medium (NM) on days 10 or 20 (Figure 2A1 and 2A2), whereas specimens cultured in osteogenic medium (OM) showed little positive staining signals in very few areas at day 10 (Figure 2B1) and slightly larger positively stained areas at day 20 (Figure 2B2). The group BMP-2 50 ng/ml showed many calcium deposits distributed over the entire section by day 10 (Figure 2C1) and even stronger positive staining by day 20 (Figure 2C2).

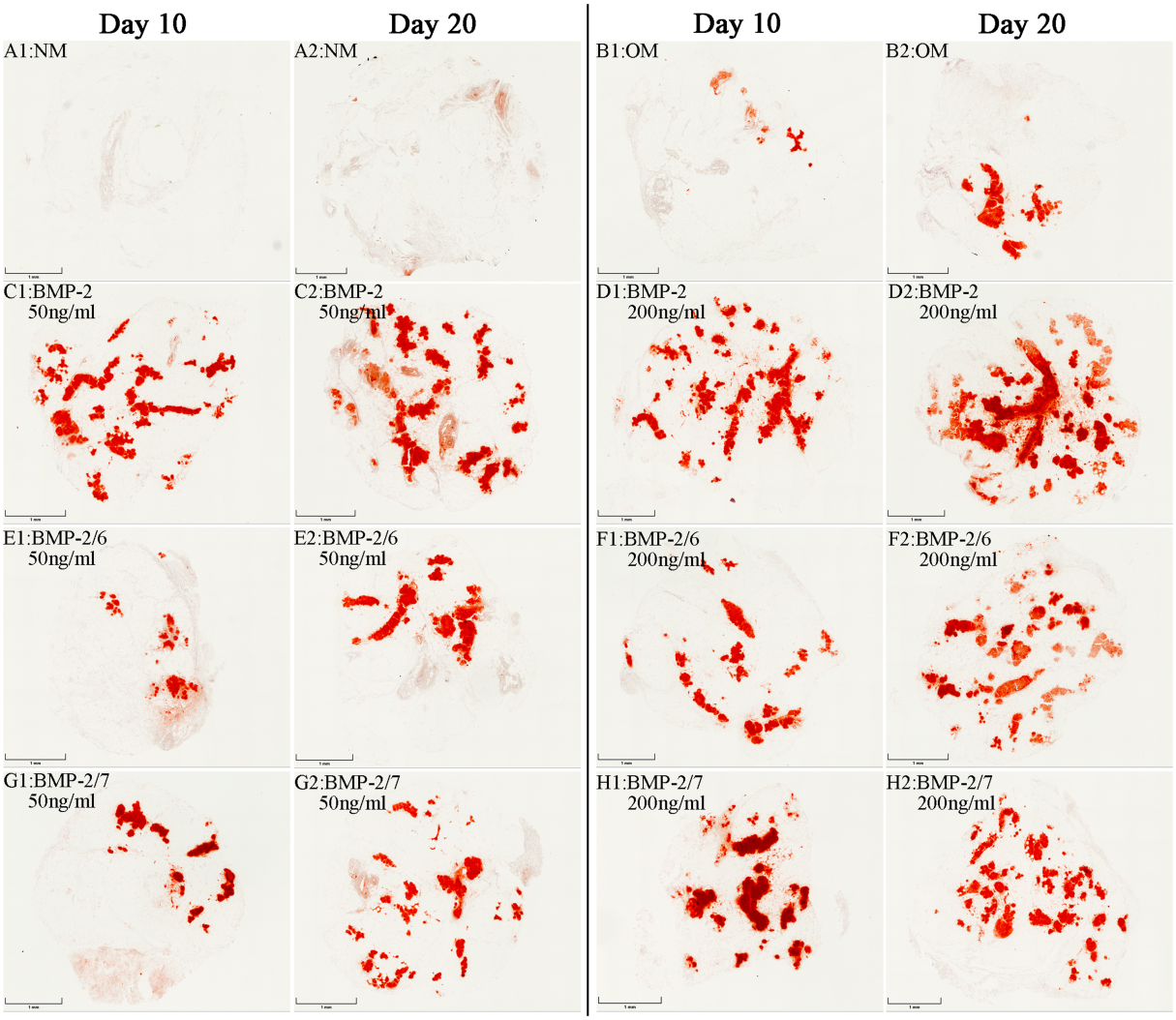

FIGURE 2 Detection of calcium deposition by alizarin red staining of histological sections, alizarin red S staining of cross-sections of adipose fragments for the detection of calcium depositions at days 10 and 20. Representative cross-sections of adipose tissue samples are shown. A1: adipose tissue cultured in normal medium (NM), day 10; A2: adipose tissue cultured in normal medium (NM), day 20; B1: adipose tissue cultured in osteogenic medium (OM), day 10; B2: adipose tissue cultured in osteogenic medium (OM), day 20; C1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2, day 10; C2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2, day 20; D1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2, day 10; D2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2, day 20; E1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/6, day 10; E2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/6, day 20; F1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/6, day 10; F2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/6, day 20; G1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/7, day 10; G2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/7, day 20; H1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/7, day 10; H2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/7, day 20. Scale bar = 1000 μm. NM, normal medium; OM, osteogenic medium

Treatment with 200 ng/ml of BMP-2 resulted in strong staining signals in many large areas of the samples on day 10 (Figure 2D1). The strongest positive staining in large areas of the tissue indicating a high content of calcium was detected in the high-concentration BMP-2 group on day 20 (Figure 2D2). The heterodimer BMP-2/6 50 ng/ml induced only a few smaller areas of calcium deposition but larger ones after 20 days of culture (Figure 2E1 and 2E2). The effect of BMP-2/6 was enhanced using the high concentration of 200 ng/ml (Figure 2F1 and 2F2). Culturing the adipose tissue in the presence of BMP-2/7 led to slightly stronger positive staining results detecting more deposits of calcium compared to BMP-2/6 at both concentrations (Figure 2G1, 2G2, 2H1 and 2H2). Adipose tissue treated with the homodimer BMP-2 showed the largest areas with strong Alizarin red S staining signals.

3.3 | Immunohistochemistry

Adipose tissue samples cultured in normal medium (NM) exhibited only minimal amounts of endogenously expressed Scl and OPN and

OCN at both time points (Figures 3A1 and 3A2, 4A1 and 4A2 and 5A1 and 5A2). Culturing adipose tissue in osteogenic medium (OM) led to an increased expression of the three proteins (Figures 3B1 and 3B2, 4B1 and 4B2 and 5B1 and 5B2). In the group BMP-2 50 ng/ml, significant amounts of OCN, OPN and Scl were detected at both time points (Figures 3C1 and 3C2, 4C1 and 4C2 and 5C1 and 5C2). Many areas distributed over the entire sections stained positive in these samples. This was clearly more pronounced compared to in the BMP-2/6 group (Figures 3E1 and 3E2, 4E1 and 4E2 and 5E1 and 5E2) and the BMP-2/7 group (Figures 3G1 and 3G2, 4G1 and 4G2 and 5G1 and 5G2) at the 50 ng/ml concentration, especially on day 10. A concentration of 200 ng/ml BMP-2 induced the highest levels of OCN, OPN and Scl expression, as indicated by dark brown staining (Figures 3D1 and 3D2, 4D1 and 4D2 and 5D1 and 5D2). This effect was clearly stronger compared to the high concentration of both heterodimers BMP-2/6 (Figures 3F1 and 3F2, 4F1 and 4F2 and 5F1 and 5F2) and BMP-2/7 (Figures 3H1 and 3H2, 4H1 and 4H2 and 5H1 and 5H2).

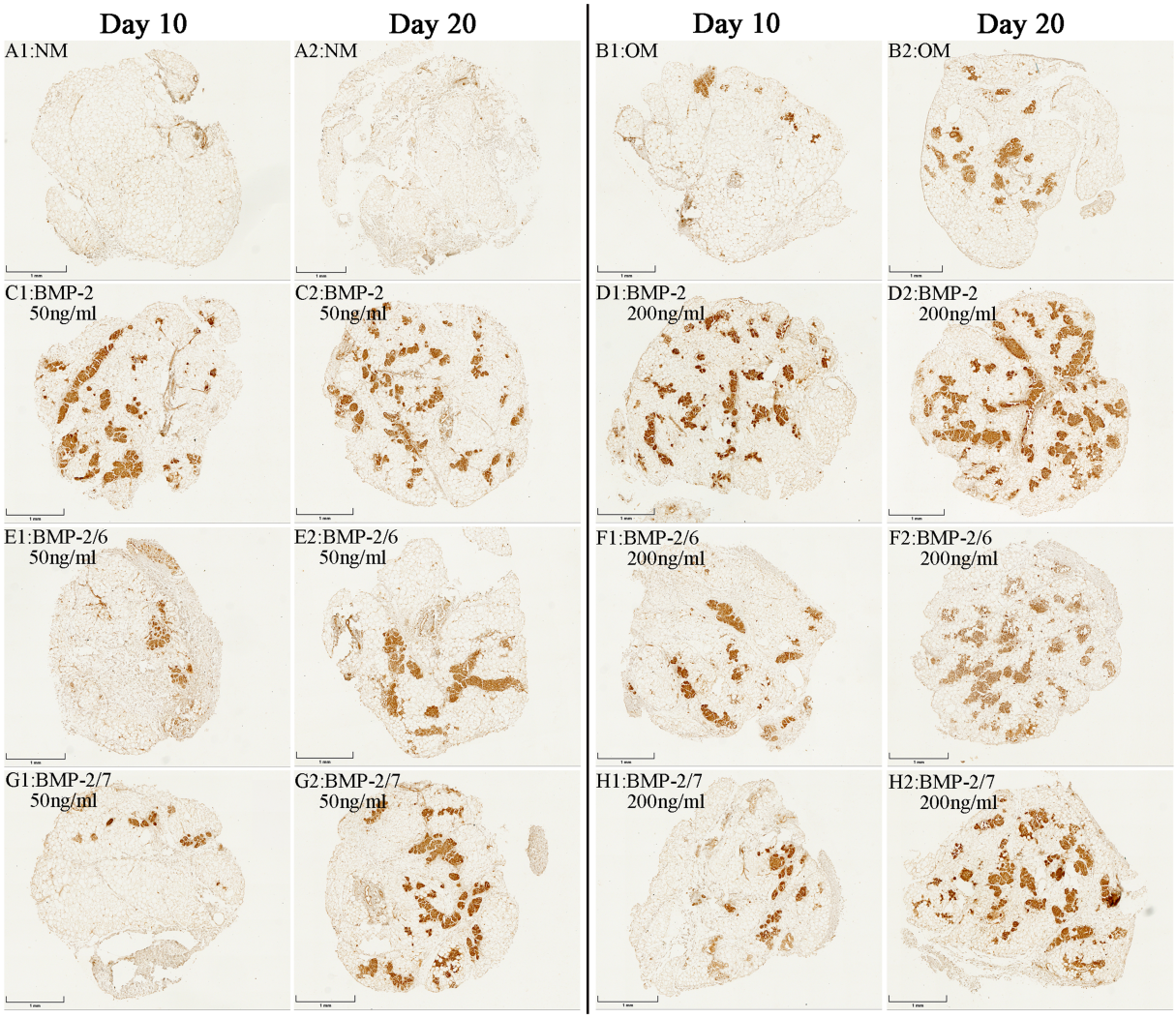

FIGURE 3 Immunohistochemistry to detect osteocalcin (OCN), cross-sections of adipose tissue samples were treated with antibodies to detect the bone-specific protein OCN. Representative specimens are shown. Scale bar = 1000 μm. A1: adipose tissue cultured in normal medium (NM), day 10; A2: adipose tissue cultured in normal medium (NM), day 20; B1: adipose tissue cultured in osteogenic medium (OM), day 10; B2: adipose tissue cultured in osteogenic medium (OM), day 20; C1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2, day 10; C2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2, day 20; D1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2, day 10; D2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2, day 20; E1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/6, day 10; E2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/6, day 20; F1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/6, day 10; F2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/6, day 20; G1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/7, day 10; G2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/7, day 20; H1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/7, day 10; H2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/7, day 20. NM, normal medium; OM, osteogenic medium

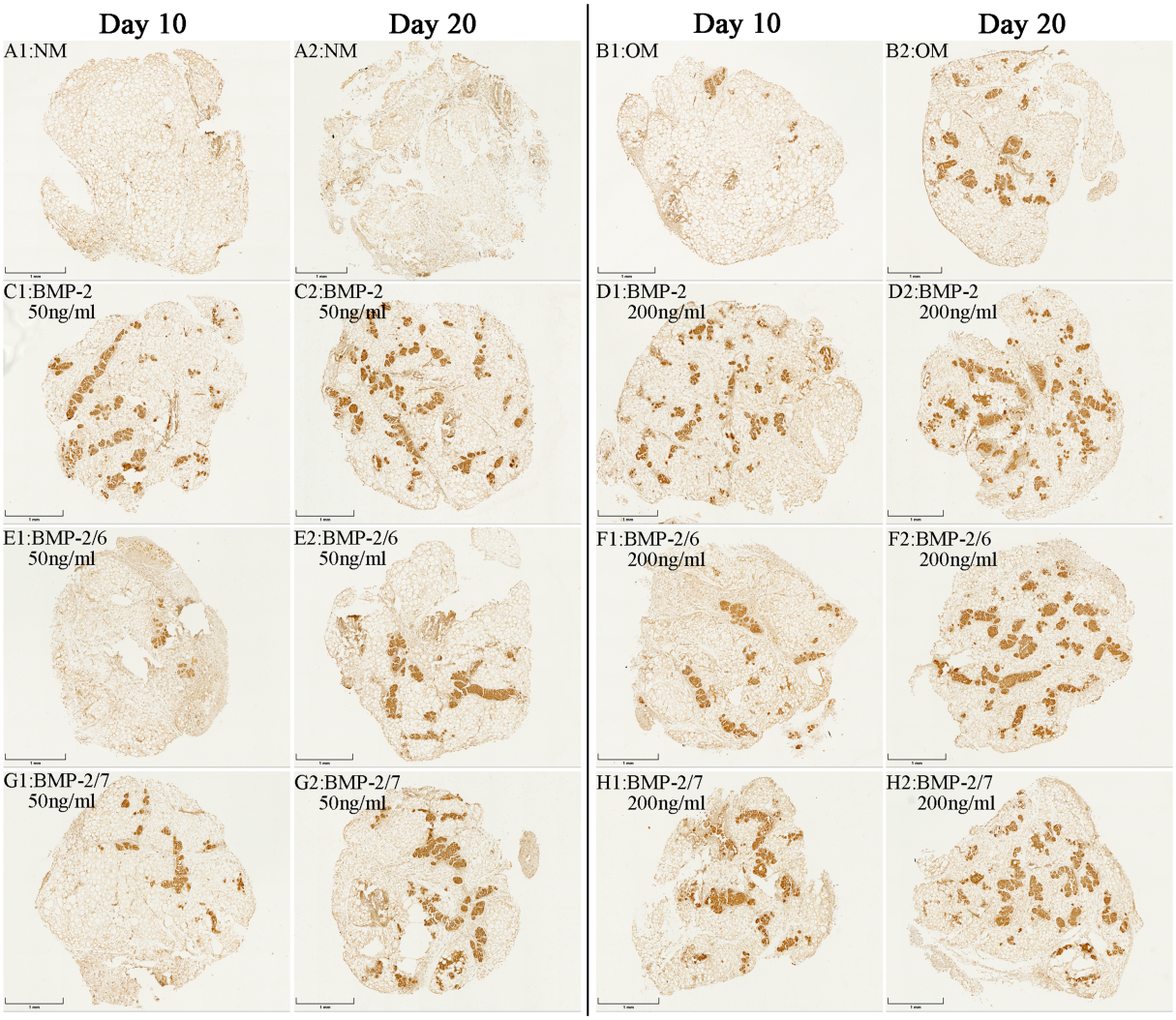

FIGURE 4 Immunohistochemistry to detect osteopontin (OPN), cross-sections of adipose tissue samples were treated with antibodies to detect the bone-specific protein OPN. Representative specimens are shown. Scale bar = 1000 μm. A1: adipose tissue cultured in normal medium (NM), day 10; A2: adipose tissue cultured in normal medium (NM), day 20; B1: adipose tissue cultured in osteogenic medium (OM), day 10; B2: adipose tissue cultured in osteogenic medium (OM), day 20; C1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2, day 10; C2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2, day 20; D1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2, day 10; D2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2, day 20; E1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/6, day 10; E2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/6, day 20; F1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/6, day 10; F2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/6, day 20; G1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/7, day 10; G2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/7, day 20; H1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/7, day 10; H2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/7, day 20. NM, normal medium; OM, osteogenic medium

3.4 | Histomorphometry

On days 10 and 20, adipose tissue receiving 200 ng/ml of BMP-2 exhibited the largest areas positively stained for calcium deposition (Figure 6A) and expression of Scl and OPN and OCN (Figure 6B, C, D). The calcium positive area of the BMP-2 200 ng/ml samples was 16.3% ± 0.8% on day 10 and 23.8% ± 1.5% on day 20, which was significantly larger than 10.6% ± 0.4% (day 10) and 15.1% ± 1.2% (day 20) in the group BMP-2/7 200 ng/ml, as well as 9.6 ± 0.8% (day 10) and 12.2% ± 1.0% (day 20) in the group BMP-2/6 200 ng/ml (Figure 6A). The calcium positive area of the group BMP-2 50 ng/ml (11,9% ± 1.7% on day 10 and 16.9% ± 0.7% on day 20) was significantly larger compared to BMP-2/6 50 ng/ml (3.7% ± 0.2% on day 10 and 8.5% ± 0.4% on day 20), BMP-2/6 200 ng/ml (9.6% ± 0.8% on day 10 and 12.2% ± 1.0% on day 20) and BMP-2/7 50 ng/ml (6.4% ± 0.4% on day 10 and 8.9% ± 0.3% on day 20) at both time points. In comparison with the group BMP-2/7 200 ng/ml (10.6% ± 0.4% on day 10 and 15.1% ± 1.2% on day 20), the mean values of the group BMP-2 50 ng/ml were slightly higher but not significantly different. A concentration of 200 ng/ml of the heterodimer

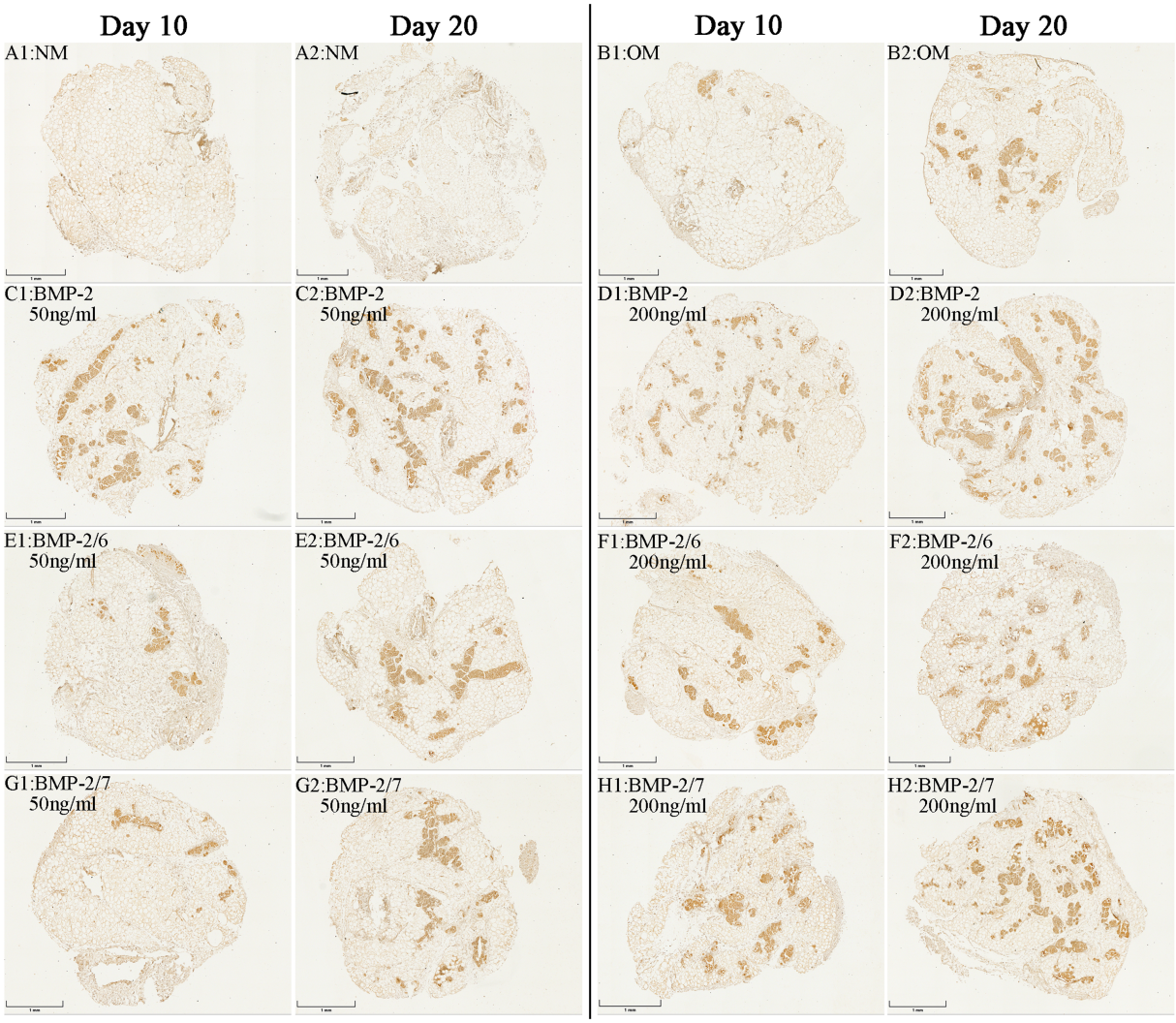

FIGURE 5 Immunohistochemistry to detect sclerostin (Scl), cross-sections of adipose tissue samples were treated with antibodies to detect the bone-specific protein Scl. Representative specimens are shown. Scale bar = 1000 μm. A1: adipose tissue cultured in normal medium (NM), day 10; A2: adipose tissue cultured in normal medium (NM), day 20; B1: adipose tissue cultured in osteogenic medium (OM), day 10; B2: adipose tissue cultured in osteogenic medium (OM), day 20; C1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2, day 10; C2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2, day 20; D1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2, day 10; D2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2, day 20; E1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/6, day 10; E2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/6, day 20; F1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/6, day 10; F2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/6, day 20; G1: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/7, day 10; G2: adipose tissue cultured in osteogenic medium + 50 ng/ml BMP-2/7, day 20; H1: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/7, day 10; H2: adipose tissue cultured in osteogenic medium + 200 ng/ml BMP-2/7, day 20. NM, normal medium; OM, osteogenic medium

BMP-2/7 elicited significantly more calcification of the adipose tissue compared to the low concentration of BMP-2/7 and both concentrations of BMP-2/6. All recombinant BMPs induced significantly more calcium deposition in the adipose tissue samples than osteogenic medium (OM) without BMP supplement (1.9% ± 0.3% on day 10 and 5.4% ± 0.7% on day 20) (Figure 6A).

The expression of OCN, OPN and Scl induced through the various culture conditions was similar. Samples cultured in osteogenic medium without BMP supplements (OM) showed significantly larger OCN-, OPN- and Scl-positive areas than the group cultured in normal medium (NM). The addition of both concentrations of all three BMPs caused an increased expression of OCN and OPN by day 20 compared to the group OM. However, on day 20, addition of the low concentrations of BMP-2/6 and BMP-2/7 did not evoke significantly increased Scl-positive areas compared to osteogenic media only. Also, on day 10, low concentrations of BMP-2/6 and BMP-2/7 did not elicit significantly larger OCN-, OPN-, and Scl-positive areas than the group OM (Figure 6B, C, D). Specimens cultured in 200 ng/ml BMP-2 showed the largest OCN- (13.3% ± 0.5% on day 10, 20.4% ± 0.6% on day 20), OPN- (11.6% ± 0.7% on day 10, 19.5% ± 0.6% on day 20) and Scl-

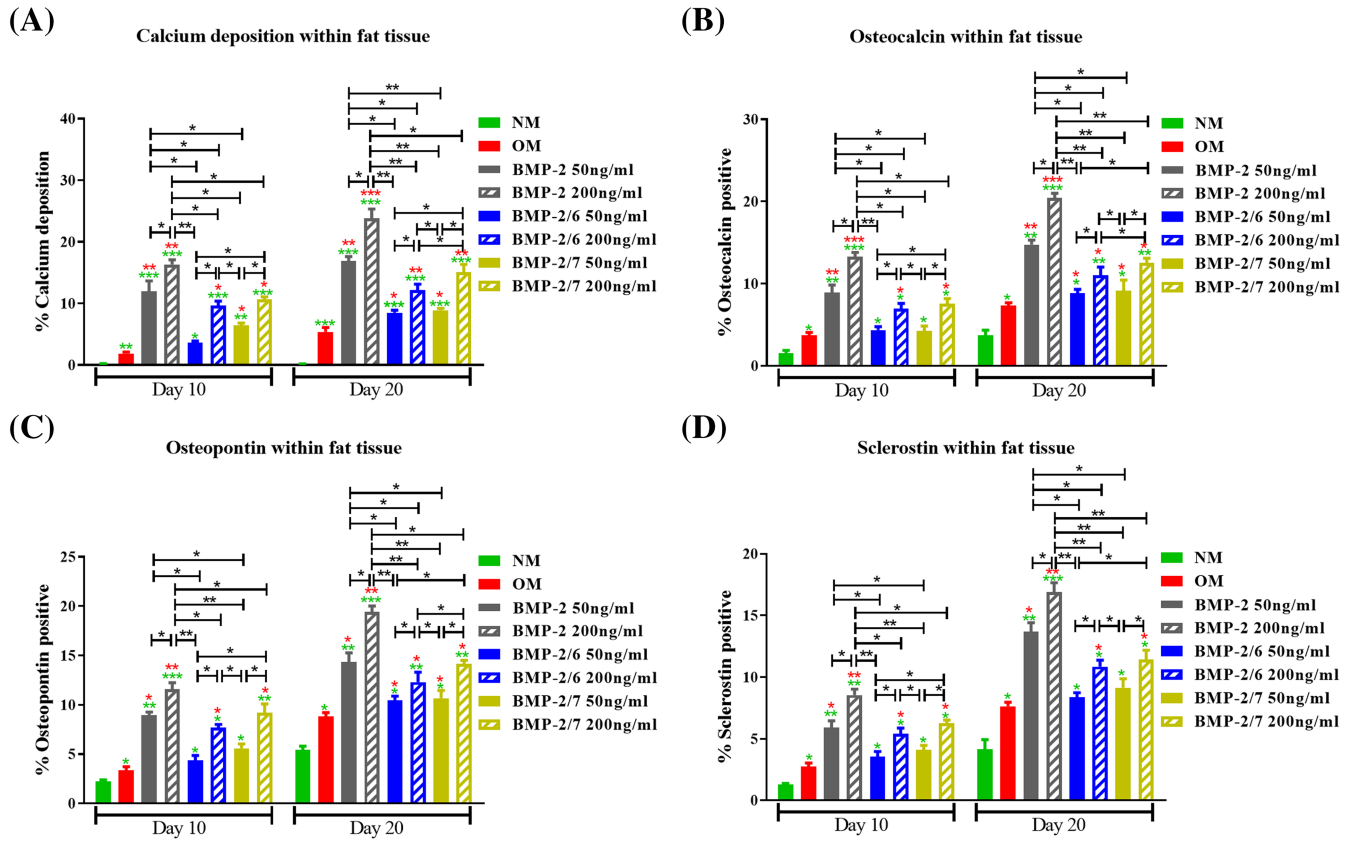

FIGURE 6 Evaluation of calcium depositions and bone-specific protein expression by histomorphometry, on days 10 and 20 of tissue culture, histomorphometry was carried out with ImageJ, version 1.6 (NIH) quantifying the mean percentage of stained area of cross-sections of adipose tissue samples. Measurements were carried out in triplicate. (A) calcium deposition; (B) OCN; (C) OPN; and (D) Scl; NM, normal medium; OM, osteogenic medium

positive (8.6% ± 0.5% on day 10, 16.9% ± 0.8% on day 20) areas. The effects of 50 ng/ml BMP-2 on the expression levels of the three proteins were not significantly different compared to 200 ng/ml BMP-2/7. However, 50 ng/ml BMP-2 caused larger OCN-, OPN- and Scl-positive areas than 50 ng/ml BMP-2/7 and both concentrations of BMP-2/6 on day 20. For example, on day 20, the OCN-positive area for the group BMP-2 50 ng/ml was 14.7% ± 0.6%, whereas 50 ng/ml of BMP-2/6 induced only 8.9% ± 0.5%, 200 ng/ml of BMP-2/6 11.1% ± 1.0% and 50 ng/ml of BMP-2/7 9.2% ± 1.3% OCN-positive area (Figure 6B, C, D).

4 | DISCUSSION

The results of the present study show that the ability of the homodimer BMP-2 to stimulate osteogenesis in adipose tissue is not enhancable using heterodimeric BMP-2/6 or BMP-2/7. BMP-2 provoked osteoinduction in adipose tissue more potently than BMP-2/6 and BMP-2/7. Our data support the use of homodimeric BMP-2 for the creation and activation of osteo-regenerative adipose tissue implants. A BMP-2 single growth factor approach to bone repair might be advantageous in terms of a potential clinical translation. The recombinant human protein BMP-2 is already in clinical use.[24]

The urgent need for an improved delivery mode of the clinically used BMP-2 is emphasized by the controversy concerning unwanted side effects related to the recombinant human protein. Recently published work resulting from the Yale University Open Data Access project reported complications using rhBMP-2 for spine fusion procedures clinically[25-27] The far supraphysiological quantities of protein necessary to elicit the desired osteogenesis as well as insufficient protein retention at the implantation site may be reasons for such unwanted effects.[8] Indeed, it was reported that the number of unwanted side effects caused by human recombinant BMP-2 is dose-dependent.[20,28–30] Gene therapy technologies may provide a solution for delivering small, physiological amounts of osteogenic molecules in a more precise fashion. The most widely used BMPs in preclinical work are BMP-2, -4, -6, -7 and -9. Several gene therapy studies demonstrated their potential to stimulate the regeneration of bone.[31-35]

To achieve faster and more robust healing, scientists explored the osteogenic effects of combinations of BMPs. For example, a dual gene therapy approach using BMP-2 and BMP-7 resulted in improved repair of cranial defects in an *in vivo* study compared to single BMP gene therapy.[16] Also, a combinatorial gene therapy using BMP-7 and

BMP-2 promoted osteogenic differentiation of progenitor cells and spinal fusion in rats more successfully compared to BMP-2 or BMP-7 alone.[36] Employing heterodimeric BMPs instead of a BMP homodimer was considered to result in superior osteogenic effectiveness and lower amounts of BMP necessary for successful bone regeneration in humans, reducing the number of unwanted side effects.[30,37] In several *in vivo* experiments, BMP-2/7 regenerated bone more effectively than the BMP homodimers.[19,20,28] BMP-2/6 and -2/7 heterodimers show enhanced osteogenic effects as a result of their affinity to both BMP receptors, type I and type II, activating Smad signaling and upregulation of osteogenic genes more effectively.[38–41] Additionally, the BMP antagonist Noggin affects the activity of heterodimeric BMPs less than that of the homodimers.[42]

However, Zhang *et al.*[43] reported that the heterodimer BMP-2/7 elicited osteogenesis in human adipose-derived cells no more potently than BMP-2 or BMP-7 homodimers. This conclusion is in agreement with the results of our experiment demonstrating that the homodimer BMP-2 was more effective stimulating osteoinduction in adipose tissue samples compared to the heterodimers. Here, the high concentration of homodimeric BMP-2 most effectively elicited osteogenesis within adipose tissue fragments, whereas its low concentration had a similar effect as the most potent heterodimer BMP-2/7 at the high concentration. The results of the present experiment, in turn, are in agreement with a previous study of our group comparing the effects of BMP-2, BMP-2/6 and BMP-2/7 on muscle tissue fragments *in vitro*.[44] In this experiment, BMP-2 had a stronger effect on gene and protein expression of bone-specific markers and calcium deposition within muscle tissue. A recent study reports that the effect of BMP heterodimers depends on the cell type.[45] Heterodimeric BMPs may have a stronger osteogenic effect on preosteoblasts[45] than on cells residing within muscle tissue[44] and adipose-derived cells.[43] This may be the reason why several *in vivo* bone healing studies revealed favorable effects of BMP heterodimers. *In vivo*, in addition to the osteoinductive effect on transplanted adipose or muscle tissue grafts, secreted BMPs may also attract and stimulate surrounding preosteoblasts within the bone lesion influencing the healing response. In the aforementioned study by Zhang *et al.*[43] the results from the *in vitro* experiment were in agreement with the results from their *in vivo* investigation. In their study using human adipose-derived cells, *in vitro* and *in vivo* osteoinduction was not enhanced using heterodimeric rhBMP-2/7 compared to homodimeric BMP-2 or BMP-7. However, scaffolds with human adipose-derived cells and BMPs were implanted subcutaneously and not into a bony lesion. This approach did not simulate the clinical scenario in which the grafted construct is implanted into an osseous lesion with BMPs affecting preosteoblasts residing in the vicinity of the lesion. Hence, a limitation of our present *in vitro* work may be that it only investigated the osteoinductive effects of homo- and heterodimeric BMPs on the adipose tissue itself. The impact on other types of cells residing in a bone defect was not taken into account. Therefore, *in vivo* experiments may still be necessary to compare and assess bone regeneration after treatment of bony defects with muscle and adipose tissue grafts in combination with homodimeric and heterodimeric BMPs.

Expedited gene therapy approaches represent an interesting alternative to conventional gene transfer strategies, allowing this to be accomplished at lower cost within a single surgical procedure. To avoid expansion and long-term cultivation of cells, a "same day" *ex vivo* gene therapy approach was developed by Virk *et al.*[13] Bone marrow-derived cells were harvested, isolated and lentivirally BMP-2 transduced within a single surgical approach. The BMP-2 producing cells repaired femoral bone defects in rats. Another expedited strategy for gene-enhanced bone regeneration represents the use of gene-activated tissue fragments. BMP-2 transduced muscle tissue[46,47] and subcutaneous adipose tissue[14] successfully healed rodent femoral defects. Especially subcutaneous adipose tissue is considered to be a very appealing and abundant source of regenerative material because it can be obtained easily with very little morbidity at the donor site.[48–50] In the fields of plastic and reconstructive surgery, adipose tissue is frequently used for rejuvenation and augmentation procedures.[51–53] Moreover, adipose tissue harbours multipotent stem cells[54,55] having the capacity for osteogenic differentiation and osseus repair.[9,56,57]

We consider the *in vitro* tissue culture system to be a suitable tool screening for promising candidate growth factors prior to generation of corresponding gene transfer vectors and *in vivo* testing. Our study results support the use of homodimeric BMP-2 in combination with adipose tissue fragments and support the further development of an expedited bone repair method. Working with the corresponding gene of the clinically approved homodimeric recombinant human BMP-2 may ease the potential translation of the technology into the clinic. However, the superior effect of homodimeric BMP-2 in combination with adipose tissue grafts should be confirmed by preclinical *in vivo* experiments applying gene transfer technologies.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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