

# Preconditioning Human Mesenchymal Stem Cells with a Low Concentration of BMP2 Stimulates Proliferation and Osteogenic Differentiation *In Vitro*

Helle Lysdahl, Anette Baatrup, Casper Bindzus Foldager, and Cody Büngrer

## Abstract

Clinical trials using bone morphogenetic protein-2 (BMP2) for bone reconstruction have shown promising results. However, the relatively high concentration needed to be effective raises concerns for efficacy and safety. The aim of this study was to investigate the osteogenic effect of an alternative treatment strategy in which human bone marrow–derived mesenchymal stem cells (hMSCs) are preconditioned with low concentrations of BMP2 for a short time *in vitro*. hMSCs in suspension were stimulated for 15 min with 10 and 20 ng/mL of BMP2. After the BMP2 was removed, the cells were seeded and cultured in osteogenic medium. The effects of preconditioning were analyzed with regard to proliferation and expression of osteogenic markers at both gene and protein level. The results were compared to those from cultures with continuous BMP2 stimulation. A significant increase in proliferation was seen with both precondition and continuous stimulation with BMP2, with no difference between the treatments. Preconditioning with BMP2 significantly increased gene expression of *RUNX2*, *COL1*, *ALP*, and *OC*, and protein levels of *COL1* and *ALP*. This was not found with continuous stimulation. The role of preconditioning with BMP2 in osteogenesis was validated by findings of increased gene expression of *SMAD1* and an increase in dual phosphorylation of ser 463 and ser 465 in the SMAD 1/5/8 pathway. We concluded that preconditioning hMSCs with BMP2 stimulates osteogenesis: proliferation with matrix secretion and matrix maturation of hMSCs. This implies that preconditioning with BMP2 might be more effective at inducing proliferation and osteogenic differentiation of hMSCs than continuous stimulation. Preconditioning with BMP2 could benefit the clinical application of BMP2 since side effects from high-dose treatments could be avoided.

**Key words:** growth factor; stem cells; tissue engineering

## Introduction

THE OSTEOINDUCTIVE POTENTIAL of recombinant bone morphogenetic protein (BMP2) has been demonstrated in a number of clinical studies.<sup>1–3</sup> Although these studies were promising, relatively high doses of BMP2 were needed to be effective (1.5–2.0 mg/mL), raising concerns about bone overgrowth and the potential subsequent risks of neural compression and oncogenic effects. High concentrations also make these treatments expensive.<sup>4</sup> Therefore, more specific assays for administration of BMP2 for bone reconstruction is necessary.

Human bone marrow–derived mesenchymal stem cells (hMSCs) have long been considered promising candidates for bone regeneration due to their capability to differentiate into osteoblasts when appropriately induced *in vitro*, allowing the use of these cells for engineering of implantable bone constructs.<sup>5,6</sup> BMP2 has been shown to stimulate the osteogenic

activity in rodent cell cultures,<sup>7–11</sup> whereas the osteogenic effects are less pronounced in human cells. Some studies indicated that continuous stimulation with BMP2 (0.1–800 ng/mL) affects the differentiation but not the proliferation of hMSCs,<sup>12–16</sup> whereas other studies did not demonstrate an osteogenic effect of 100 ng/mL BMP2.<sup>17,18</sup>

Preconditioning strategies in stem cell therapy is an emerging research area, and a number of studies have shown increased regenerative and repair potentials of preconditioned cells (reviewed by Yu et al.<sup>19</sup>). It was previously shown that preconditioning with 10 ng/mL BMP2 for 15 min stimulates osteogenic differentiation of adipose stem cells derived from goats.<sup>20</sup> Preconditioning of hMSCs with a low concentration of BMP2 could be beneficial for clinical application since side effects from the high-dose treatments will be avoided and the treatment will be less expensive. Therefore, the aim of this study was to investigate the proliferative and osteogenic effects of hMSCs preconditioned for

15 min with 10 and 20 ng/mL of BMP2 compared with continuous BMP2 stimulation. We hypothesized that preconditioning for a short time with a low concentration of BMP2 would stimulate the proliferation and/or osteogenic differentiation of hMSCs.

## Materials and Methods

### Cell culture

Bone marrow-derived human hMSCs (PT-2501, lot 1F3284, two different clones, Lonza; female donor, 21 years, 10.94–11.42 population doublings, in suspension 14,000 cells/mL) were preconditioned in Dulbecco's modified Eagle's medium (DMEM) without phenol red (21063, Gibco, Invitrogen)<sup>21</sup> supplemented with 10% fetal bovine serum (FBS) and either 0, 10, or 20 ng/mL BMP2 Recombinant Human Protein (PHC7145, Gibco, Life Technologies) for 15 min at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells were washed once with phosphate-buffered saline (PBS) before seeding in 96-well (83.1835, Sarstedt), 24-well (83.1836, Sarstedt), or 16-well glass chamber slides (178599, Nunc), 8000 cells/cm<sup>2</sup> in osteogenic differentiation medium consisting of DMEM supplemented with 10% FBS, 100 nM dexamethasone (D2915, Sigma), 10 mM  $\beta$ -glycerophosphate (G9891, Sigma), 50  $\mu$ M L-ascorbic acid-2 phosphate (A8960, Sigma), and 10<sup>-8</sup> M 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (D1530, Sigma). For continuous BMP2 stimulation the osteogenic medium was further supplemented with either 10 or 20 ng/mL (hereafter referred to as 10 ng/mL+ and 20 ng/mL+, respectively) throughout the culture period. The cells were cultured for up to 11 days at 37°C, in a humidified atmosphere of 5% CO<sub>2</sub>. Media were changed twice a week.

### Cell viability

Cell viability was measured using an XTT assay (11465015001, Roche Applied Science) according to the manufacturer's instructions. Briefly, cells grown for 2, 4, and 7 days were incubated with the XTT labeling mixture for 3 h. The con-

version of yellow tetrazolium salt XTT to orange formazan was measured at 490/650 nm using a Victor<sup>3</sup> Multilabel Counter (Perkin Elmer).

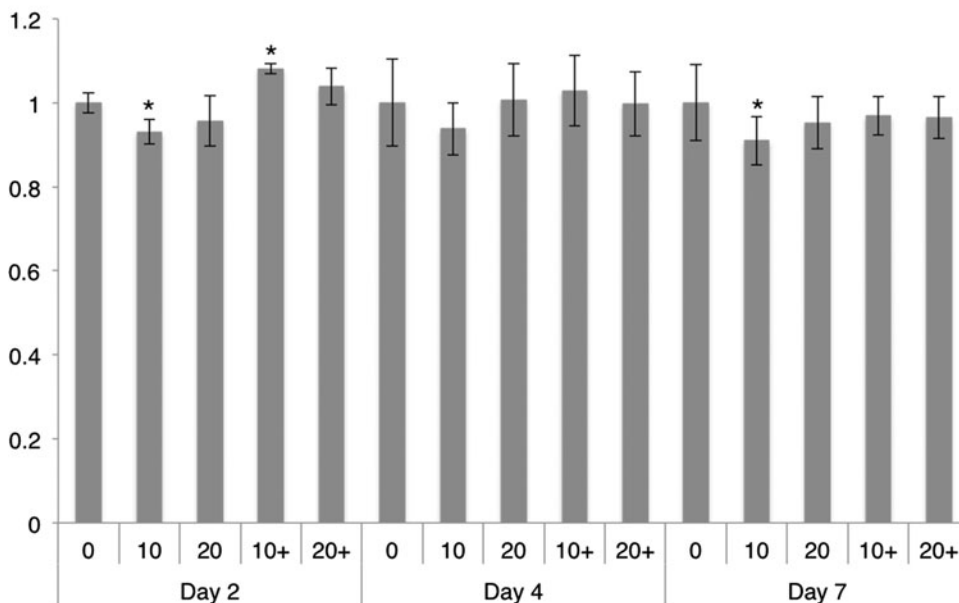
### Cell proliferation

Cell proliferation was measured using a methylene blue assay.<sup>22</sup> Briefly, cells grown for 2, 4, and 7 days were fixed by methanol before stained with 1‰ (w/v) methylene blue in 0.01 M borate buffer (pH 8.5). Blue staining due to electrostatic binding of methylene-blue to negatively charged groups within the cells was eluted by 1:1 (v/v) ethanol and 0.1 M HCl. The absorbance was measured at 650 nm using a Victor<sup>3</sup> Multilabel Counter.

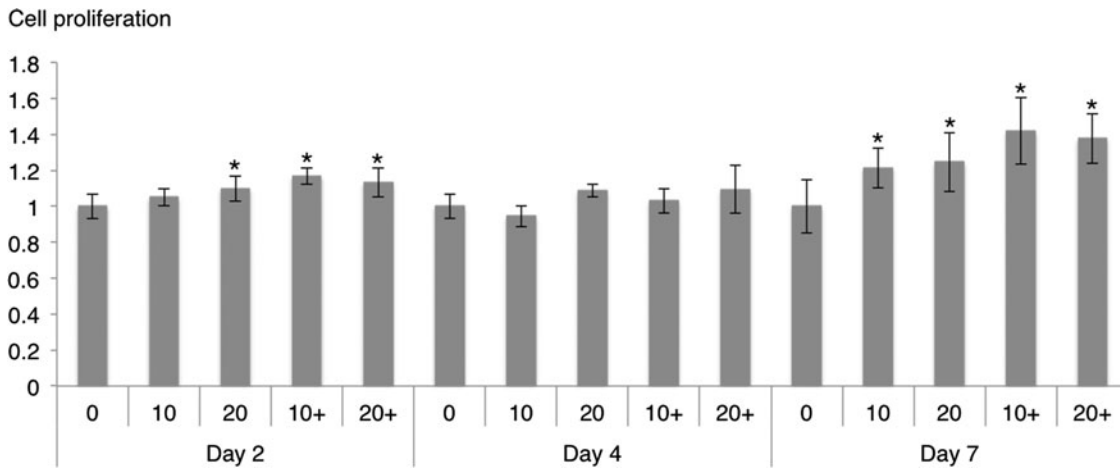
### Total RNA extraction and RT-qPCR

Total RNA was extracted after 0 and 6 h, 1, 2, 7, and 11 days in osteogenic cultures with the GenElute<sup>TM</sup> Mammalian Total RNA Miniprep Kit (RTN 350, Sigma-Aldrich) according to the procedure of the manufacturer. RNA concentration and purity were spectrophotometrically determined using an IMPLEN NanoPhotometer<sup>TM</sup> (VWR Bie & Berntsen) according to manufacturer's instructions. RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies) according to the procedure of the manufacturer. The RNA samples were treated with DNase I (AM2222, Ambion) and converted into complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (4368813, Applied Biosystems). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems) using TaqMan<sup>®</sup> Fast Universal PCR Master Mix (4366073, Applied Biosystems) and TaqMan<sup>®</sup> Gene Expression Assays (4331182, Applied Biosystems) with the following assays: runt-related transcription factor 2 (*RUNX2*) Hs00231692\_m1, collagen type I alpha 1 (*COL1*) Hs00164004\_m1, alkaline phosphatase (*ALP*) Hs00758162\_m1, bone gamma-carboxylglutamate protein

### Cell viability



**FIG. 1.** Cell viability of human mesenchymal stem cells (hMSCs) cultured for 2, 4, and 7 days after 15 min exposure to either 0, 10, 20 ng/mL bone morphogenetic protein-2 (BMP2) or continuous stimulation with BMP2 throughout the culture period (10 ng/mL+ and 20 ng/mL+, respectively). Vertical axis represents the relative absorbance at 490 nm after normalization of the different BMP2 exposures to 0 ng/mL at each time point. Horizontal axis represents the different BMP2 exposures at different time points. Data are expressed as mean  $\pm$  SD ( $n=6$ ). \*Significant difference between 0 ng/mL and exposure to BMP2 within the given time point,  $p < 0.05$ .



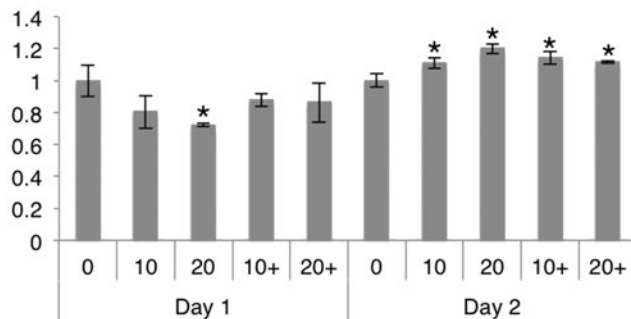
**FIG. 2.** Cell proliferation of hMSCs cultured for 2, 4, and 7 days after 15 min exposure to either 0, 10, or 20 ng/mL BMP2 or continuous stimulation with BMP2 throughout the whole culture period (10 ng/mL + and 20 ng/mL +, respectively). Vertical axis represents the relative absorbance at 650 nm after normalization of the different BMP2 exposures to 0 ng/mL at each time point. Horizontal axis represents the different BMP2 exposures at different time points. Data are expressed as mean  $\pm$  SD ( $n=6$ ). \*Significant difference between 0 ng/mL and exposure to BMP2 at the given time point,  $p < 0.05$ .

(osteocalcin [OC] Hs01587813\_g1, SMAD family member 1 (SMAD1) Hs00195432\_m1, SMAD family member 5 (SMAD5) Hs00195437\_m1, and SMAD family member 8 (SMAD8) Hs00195441\_m1. Standard enzyme and cycling conditions for the 7500 Fast System were used. Amplicon

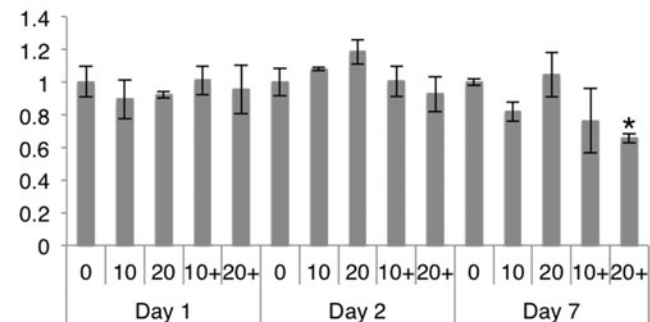
size was  $< 130$  bp for all primer sets to maximize the amplification efficiency. Template cDNA corresponding to 8 ng of RNA was added to each PCR amplification and each biological sample was run in technical duplicates for each gene. Data analysis was performed using 7500 Fast System

### Gene expression

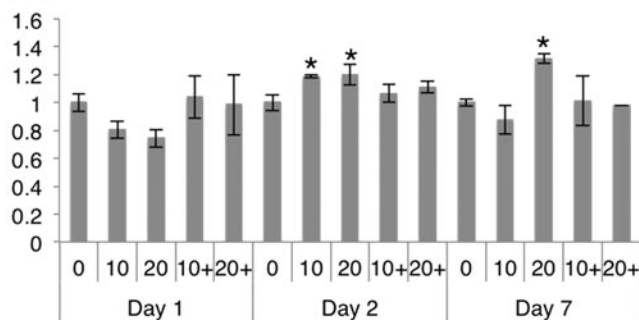
#### RUNX2



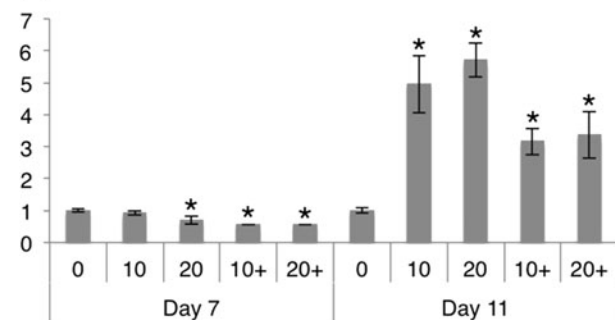
#### ALP



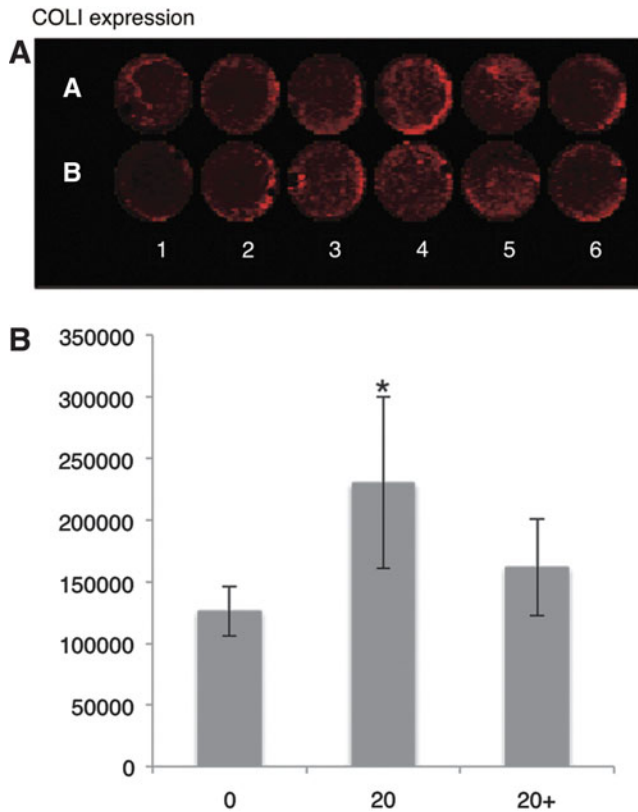
#### COL1



#### OC



**FIG. 3.** Gene expression of *RUNX2*, *COL1*, *ALP*, and *OC* after 15 min exposure to either 0, 10, or 20 ng/mL BMP2 or continuous stimulation with BMP2 throughout the culture period (10 ng/mL + and 20 ng/mL +, respectively). Vertical axes represent the BestKeeper relative gene expression normalized to 0 ng/mL BMP2 at each time point. Horizontal axis represents the different BMP2 exposures at different time points. Data are expressed as mean  $\pm$  SD ( $n=6$ ). \*Significant difference between 0 ng/mL and exposure to BMP2 at the given time point,  $p < 0.05$ . *RUNX2*, runt-related transcription factor 2; *COL1*, collagen type I alpha 1; *ALP*, alkaline phosphatase; *OC*, osteocalcin.



**FIG. 4.** Protein expression of COLI at day 4 after 15 min exposure to 0 or 20 ng/mL BMP2 or continuous stimulation with 20 ng/mL BMP2 throughout the culture period (20 ng/mL+) visualized and quantitative determined by immunostaining. (A) Picture of the slide after scanning by Odyssey<sup>®</sup>Sa Infrared Imaging: 0 ng/mL BMP2: A1, A2, B2, negative control (no primary antibody added): B1, 20 ng/mL: A3, A4, B3, B4, 20 ng/mL+: A5, A6, B4, B6. (B) Quantitative evaluation of the scanned slide. Vertical axis represents infrared signal subtracted the signal from the negative control. Horizontal axis represents the different BMP2 exposures at day 4. Data are expressed as mean ± SD (*n*=4). \*Significant difference between 0 ng/mL and exposure to BMP2, *p*<0.05.

Sequence Detection Software version 1.3 (Applied Biosystems). Based on BestKeeper,<sup>23</sup> values were normalized to ubiquitin C Hs00824723\_m1 and β-2-microglobulin Hs99999907\_m1.

*Alkaline phosphatase activity assay*

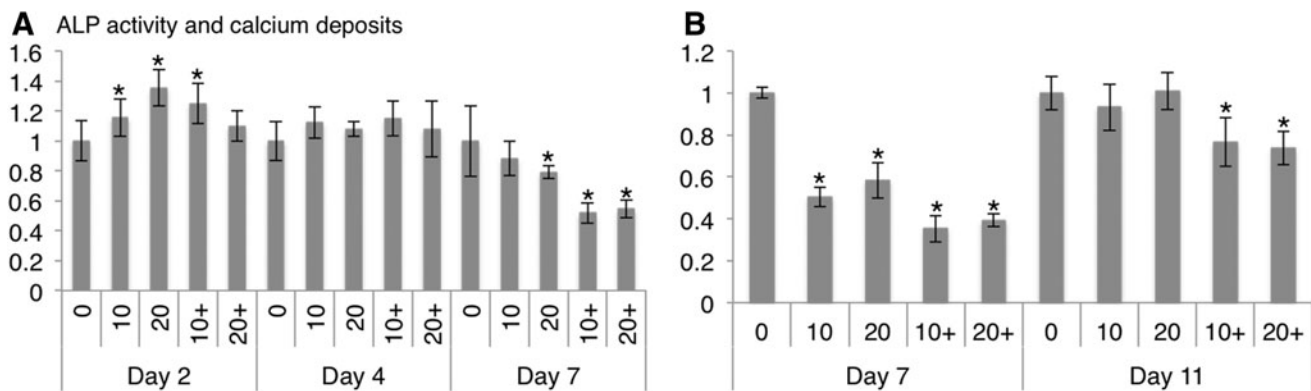
Alkaline phosphatase (ALP) activity was determined using the ALP activity assay (A9226 and P5994, Sigma) according to the procedure of the manufacturer. Briefly, the conversion of colorless *p*-nitrophenyl phosphate to colored *p*-nitrophenol was measured after 2, 4, and 7 days of culture in osteogenic media at 405/650 nm using a Victor<sup>3</sup> Multilabel Counter.

*Calcium deposition*

Calcium deposits were determined after 7 and 11 days of culture by alizarin red (AZR) staining. Cells were fixed in 70% ethanol for 60 min. After being rinsed with double distilled water (ddH<sub>2</sub>O), the cells were stained with 0.2% AZR (A5533, Sigma) in 2% ethanol for 15 min, rinsed five times with ddH<sub>2</sub>O, and dried at room temperature overnight. The cells were destained with 5% sodium dodecyl sulfate in 0.5 M HCl, and the AZR concentration was determined at 405/600 nm using a Victor<sup>3</sup> Multilabel Counter.

*Immunostainings*

For detection of COLI and phosphorylation of SMAD 1/5/8 (Ser 463/Ser 465) (p-SMAD1/5/8) cells were fixed with 70% ethanol after 24 h and 4 days of culture in osteogenic medium. After being rinsed with PBS, the cells were incubated with 10% bovine serum albumin for 60 min at room temperature before being incubated overnight with COLI (sc-8784-R, Santa Cruz Biotechnology, Inc.) or p-SMAD1/5/8 (sc-12353-R, Santa Cruz Biotechnology, Inc.), dilution 1:100, 4°C. After being rinsed with PBS, the cells were incubated for 60 min with a biotinylated antibody (E0432, DAKO), dilution 1:300. They were again rinsed with PBS, then incubated 120 min with streptavidin and Alexa Fluor<sup>®</sup> 700 conjugate (S-21383, Life Technologies) for detection of COLI or streptavidin and



**FIG. 5.** ALP activity and calcium deposits relative to cell number after 15 min exposure to either 0, 10, or 20 ng/mL BMP2 or continuous stimulation with BMP2 throughout the culture period (10 ng/mL+ and 20 ng/mL+, respectively). Vertical axes represent (A) the relative ALP activity after normalization of the different BMP2 exposures to 0 ng/mL at each time point and (B) the relative concentration of alizarin red (AZR) after normalization of the different BMP2 exposures to 0 ng/mL at each time point. Horizontal axes represent the different BMP2 exposures at different time points. Data are expressed as mean ± SD (*n*=6). \*Significant difference between 0 ng/mL and exposure to BMP2 at the given time point, *p*<0.05.

Alexa Fluor<sup>®</sup> 488 conjugate (S-11223, Life Technologies) for detection of p-SMAD1/5/8, dilutions 1:500. After being rinsed with PBS, the cells were dried overnight at 4°C. Quantitative detection of COLI was then performed using Odyssey<sup>®</sup> Sa Infrared Imaging (Licor Biosciences) and Image Studio software (version 3.1.4) (Licor Biosciences). Cells for p-SMAD1/5/8 evaluation were mounted in glycerol mounting medium and visualized by fluorescence microscopy.

### Statistics

Data were analyzed using Stata Statistical Software, release 12.0 (StataCorp LP, College Station, TX). Data from the different experiments were normalized to 0 ng/mL BMP2 at each time point, and normal distribution was checked by QQ-plots and the assumption of variance homogeneity by Bartlett's test. At each time point the different BMP2 exposure groups were analyzed by one-way ANOVA. The significance level was  $p < 0.05$ .

## Results

### Analysis of cell viability and proliferation

The concentrations used in our study were determined by pilot studies in which proliferation was increased at day 2 after 15-min exposure to 10, 20, 50, 100, 200, and 300 ng/mL BMP2. Concentrations of 50–300 ng/mL were not significantly different from 20 ng/mL (data not shown). Therefore, 10–20 ng/mL concentrations were chosen for further examinations. Cell viability was decreased at days 2 and 7 by 10 ng/mL and increased at day 2 by 10 ng/mL+ BMP2 (Fig. 1). Proliferation was significantly increased at day 2 by 20 ng/mL, 10 ng/mL+, and 20 ng/mL+ BMP2 and at day 7 by all BMP2 treatments (Fig. 2).

### Examination of genes involved in osteogenic differentiation

*RUNX2* was significantly decreased at day 1 by 20 ng/mL BMP2. At day 2 all BMP2 exposures increased *RUNX2* expression. *COLI* was significantly increased at day 2 by 10 and 20 ng/mL BMP2 and at day 7 by 20 ng/mL BMP2. *ALP* was decreased at day 7 by 20 ng/mL+ BMP2. *OC* was significantly decreased at day 7 by 20 ng/mL, 10 ng/mL+, and 20 ng/mL+ BMP2. At day 11 all BMP2 exposures increased *OC* expression (Fig. 3).

### Analysis of COLI protein expression

At day 4 more COLI was present when the cells were stimulated with 20 ng/mL and 20 ng/mL+ BMP2 (Fig. 4A: A3, A4, B3, B4 [20 ng/mL] and A5, A6, B5, B6 [20 ng/mL+], respectively) compared to 0 ng/mL BMP2 (Fig. 4A: A1, A2, B2). Quantitative evaluation revealed that 20 ng/mL BMP2 increased the expression of COLI significantly (Fig. 4B).

### Analysis of ALP activity and calcium deposits

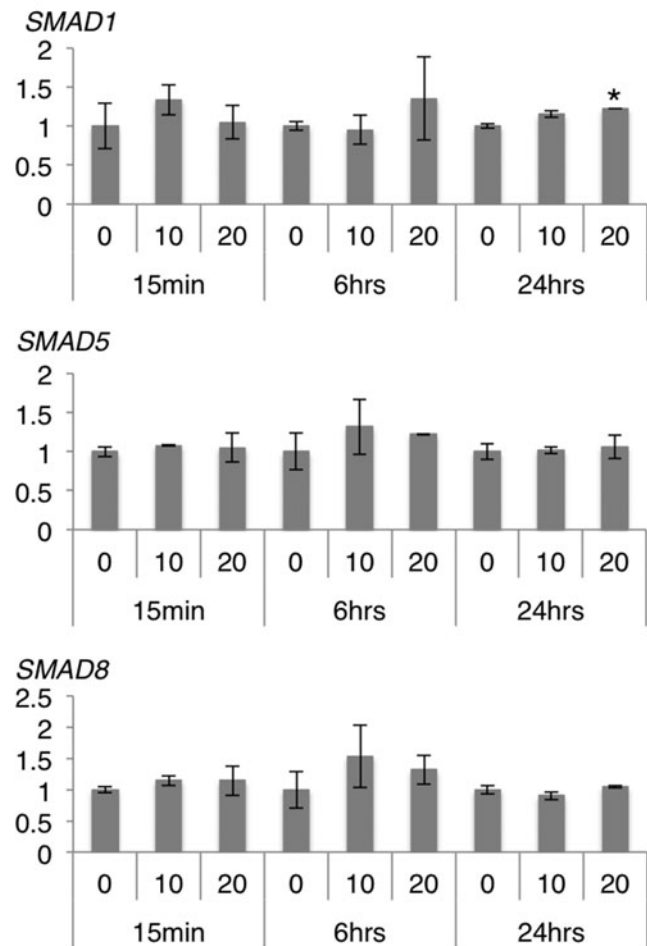
Since BMP2 affected the proliferation of the hMSCs, the ALP activity and calcium deposits (AZR) were analyzed with regard to the cell number and determined relative to methylene blue assay values. At day 2, 10 ng/mL, 20 ng/mL, and 10 ng/mL+ BMP2 significantly increased the ALP activ-

ity. At day 7, 20 ng/mL, 10 ng/mL+, and 20 ng/mL+ BMP2 significantly decreased the ALP activity (Fig. 5A). At day 7, all BMP2 exposures significantly decreased the AZR. At day 11, 10 ng/mL+ and 20 ng/mL+ significantly decreased the AZR (Fig. 5B).

### Examination of the BMP2 pathway—involvement of the SMAD family members 1, 5, and 8

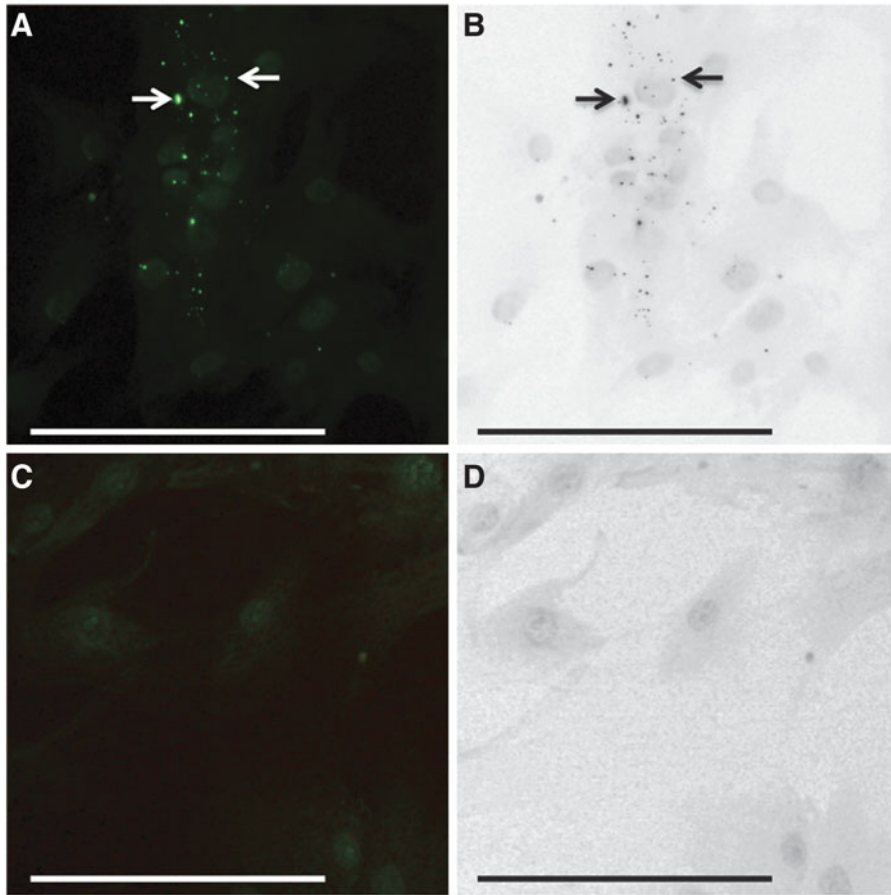
Evaluation of the gene expressions of the *SMAD* family members 1, 5, and 8 revealed that *SMAD1* was significantly increased after 24 h by 20 ng/mL BMP2. *SMAD5* and *SMAD8* was unaffected at the chosen time points (Fig. 6). At the protein level 20 ng/mL BMP2 increased Ser 463 and Ser 465 dual phosphorylation of SMAD 1/5/8 compared to 0 ng/mL BMP2 after 24 h (Fig. 7).

### Gene expression



**FIG. 6.** Gene expression of *SMAD1*, *SMAD5*, and *SMAD8* after 15 min exposure to either 0, 10, or 20 ng/mL BMP2. Vertical axes represent the BestKeeper relative gene expression normalized to 0 ng/mL BMP2 at each time point. Horizontal axis represents the different BMP2 exposures at different time points. Data are expressed as mean ± SD (n=6). \*Significant difference between 0 ng/mL and exposure to BMP2 at the given time point,  $p < 0.05$ . *SMAD1*, *SMAD5*, *SMAD8*: SMAD family members 1, 5, and 8, respectively.

p-SMAD 1/5/8 expression



**FIG. 7.** Expression of Ser 463 and Ser 465 dual phosphorylation of SMAD 1/5/8 after 15 min exposure to 0 or 20 ng/mL BMP2 following 24 h of cultivation visualized by immunostainings. (A) 20 ng/mL BMP2. (C) 0 ng/mL BMP2. For better visualization the original pictures of green staining were converted into black/white tones and inverted: (B) 20 ng/mL BMP2 and (D) 0 ng/mL BMP2. Arrows indicate positive staining. Scale bar = 150  $\mu$ m. Original magnifications:  $\times 10$ .

## Discussion

In the present study we found that preconditioning hMSCs with BMP2 using 15 min exposure to 20 ng/mL BMP2 increased the proliferation and osteogenic differentiation of hMSCs, while continuous exposure to a similar concentration only increased the proliferation and did not initiate osteogenic differentiation. Our results were supported by data obtained from several outcome measures: increased gene expression of the osteogenic markers *RUNX2*, *COL1*, and *OC*; increased protein expression of *COL1* and ALP activity; and involvement of the SMAD signaling pathway.

Previously no proliferative effect was found in hMSCs continuously stimulated with BMP2 (0.1–200 ng/mL).<sup>13,15,16,24</sup> In another study, a decrease in proliferation was found in hMSCs after continuous stimulation with 100 ng/mL BMP2 for 1 day.<sup>14</sup> In correlation with our study it has previously been shown that 15 min exposure to 10 ng/mL BMP2 increased the proliferation of MSCs derived from goat adipose tissue after 14 days of culture.<sup>20</sup>

*RUNX2* is the earliest transcription factor expressed during osteogenic differentiation and can be induced in C3H10T1/2 cells by BMP2.<sup>25</sup> Similar to our study, it has previously been shown that 15 min exposure to 10 ng/mL BMP2 increased *RUNX2* in adipose-derived MSCs from goats.<sup>20</sup> In an immortalized hMSC line, it was found that 100 ng/mL BMP2 increased *RUNX2* after 1 h of stimulation.<sup>16</sup> Continuous stimulation with 250 ng/mL BMP2 did

not increase the expression of *RUNX2*.<sup>26</sup> One of the first products formed during osteogenesis is *COL1* and ALP. To our knowledge, the effect of preconditioning with a low concentration of BMP2 on *COL1* expression has not been described before. The effect of continuous stimulation with BMP2 on *COL1* expression of hMSCs is not clear. Two studies have described that 25–100 ng/mL BMP2 increased both the mRNA and protein levels in an immortalized hMSC line and hMSCs.<sup>14,16</sup> Other studies have shown that 250 ng/mL<sup>26</sup> and 100 ng/mL<sup>15</sup> did not increase the expression of *COL1* in hMSCs. Similar to our study, it was previously shown that short-term exposure to 10 ng/mL BMP2 increased the ALP of MSCs derived from goat adipose tissue.<sup>20</sup> The effect of continuous stimulation with BMP2 on ALP expression is contradictory. Studies have shown that 100 ng/mL BMP2 increased ALP in an immortalized hMSC line and hMSCs.<sup>14–16</sup> In other studies, no stimulatory effect on ALP was observed by addition of 100 ng/mL BMP2.<sup>13,18</sup> The mineralization effect of short-term exposure to BMP2 has not been described previously. It has been shown that continuous stimulation with 100 ng/mL BMP2 increased both the expression of *OC* and calcium deposition in an immortalized hMSC line and hMSCs.<sup>13–16</sup> In our data there was no correlation between *OC* expression and calcium deposits. Since *OC* is expressed by mineralizing osteoblasts, we also expected the level of calcium deposition to be increased by the short-term exposure to BMP2 at day 11. We speculated whether this might be due to limitations in the *in vitro* set-

up that restrict the cells from mineralizing; for instance, if the medium change twice a week was insufficient. Collectively, our data indicate that short-term exposure to 20 ng/mL BMP2 stimulates osteogenesis by increasing the proliferation and expression of *RUNX2*, *COL1*, and *ALP*, indicating the beginning of deposition and maturation of extracellular matrix.

BMPs signal via heteromeric complexes of type I and type II serine-threonine kinase receptors, which propagate downstream signaling mainly via the SMAD 1/5/8 pathway. Upon receptor mediate phosphorylation of these SMADs, they associate with SMAD4 and translocate to the nucleus where they stimulate transcription of target genes.<sup>27</sup> We therefore investigated whether the stimulating osteogenic effect of preconditioning with 20 ng/mL BMP2 found in this study could involve these signaling pathways. We found that the expression of *SMAD1* and phosphorylated SMAD 1/5/8 was increased. The involvement of the SMAD signaling pathway during BMP2 stimulation of hMSCs has not clearly been described before. One study has shown that SMAD signaling was activated by 50 ng/mL BMP2.<sup>28</sup> Another study showed that PI3-K signaling was required for BMP2-induced osteogenesis either downstream or independent of the SMAD signaling pathway.<sup>17</sup> In rodent cell cultures, it has previously been described that phosphorylation and activation of SMAD 1/5/8 was followed by expression of *RUNX2* which resulted in the expression of several proteins critical for bone formation such as *COL1*, *ALP*, and *OC*.<sup>25,29</sup> Together our data correlate with these studies. Therefore, we believe that the SMAD signaling pathway is involved in the proliferative and osteogenic response of hMSCs after preconditioning with 20 ng/mL BMP2.

The discrepancies in the osteogenic effects of hMSCs stimulated with BMP2 might result from the large variations in the experimental set-ups among the previously performed studies, especially in terms of which osteogenic stimulants have been added together with BMP2 that might influence the effect of BMP2. It has previously been described that BMP2 enhances dexamethasone-induced osteogenesis.<sup>13,18</sup> The effect of BMP2 detected in the present study was achieved without any other supplements. In pilot studies we tested the effect of short-term exposure to BMP2 in media containing dexamethasone,  $\beta$ -glycerophosphate, L-ascorbic acid-2 phosphate, and  $1\alpha,25(\text{OH})_2\text{D}_3$  and found no osteogenic effect of BMP2 (data not shown). Therefore, it seems that the effect of BMP2 on hMSCs might be very sensitive to other supplements being in the medium.

In the present study, we used two clones of commercially available hMSCs, from a 21-year-old female donor, purchased from Lonza. Whether the effect of preconditioning with 20 ng/mL BMP2 on proliferation and osteogenic differentiation is reproducible between donors needs to be explored further. We also only examined the involvement of SMAD signaling pathways. Studies suggest a existing relationship between BMPs and Wnt signaling pathway (reviewed by Rosen<sup>30</sup>) and cross-talks between receptor tyrosine kinases and BMP signaling<sup>28</sup> that may affect the differentiation of hMSCs into osteoblasts.

The "gold standard" in restoration of larger bone defects has for decades been autografting. The procedure often causes donor site morbidity and increases the risk of infection.<sup>31</sup> An alternative to bone grafts is BMP2. The U.S.

Food and Drug Administration and the European Medicines Agency have approved it for clinical use for specific applications in spinal fusion and fracture healing.<sup>32</sup> However, the high doses needed for it to be effective have raised concerns about safety because of serious complications observed in some cases.<sup>33</sup> Thus, finding a strategy to reduce the dose of BMP2 for clinical practice will be of great importance. In the present study, we demonstrated a preconditioning strategy of hMSCs with a low concentration of BMP2 *in vitro*. This finding may have potential applications in spinal fusion surgery, for which autologous hMSCs could be isolated, preconditioned with BMP2, seeded on a scaffold such as the collagen sponge used in the INFUSE<sup>®</sup> bone graft,<sup>32</sup> and subsequently implanted in the patient. Such a strategy could benefit the clinical application of BMP2 since the delivery of a low concentration of BMP2 is controlled to affect only the hMSCs stimulated *ex vivo* whereby side effects from the high-dose treatments could be avoided.

### Acknowledgments

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### Author Disclosure Statement

No competing financial interests exist.

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#### Abbreviations Used

ALP = alkaline phosphatase  
 AZR = alizarin red  
 BMP2 = bone morphogenetic protein-2  
 cDNA = complementary DNA  
 ddH<sub>2</sub>O = double-distilled water  
 DMEM = Dulbecco's modified Eagle's medium  
 FBS = fetal bovine serum  
 hMSC = human mesenchymal stem cell  
 RT-qPCR = real-time quantitative polymerase chain reaction