

# HGF inhibits BMP-induced osteoblastogenesis: possible implications for the bone disease of multiple myeloma

Therese Standal,<sup>1</sup> Niels Abildgaard,<sup>2</sup> Unn-Merete Fagerli,<sup>1</sup> Berit Stordal,<sup>1</sup> Øyvind Hjertner,<sup>1,3</sup> Magne Borset,<sup>1,4</sup> and Anders Sundan<sup>1</sup>

<sup>1</sup>Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, Trondheim, Norway;

<sup>2</sup>Department of Haematology, Odense University Hospital, Denmark; <sup>3</sup>Department of Hematology and <sup>4</sup>Department of Immunology and Transfusion Medicine, St Olav University Hospital, Trondheim, Norway

**The bone disease in multiple myeloma is caused by an uncoupling of bone formation from bone resorption. A key difference between patients with and patients without osteolytic lesion is that the latter have fewer and less active osteoblasts. Hepatocyte growth factor (HGF) is often produced by myeloma cells and is found at high concentrations in the bone marrow of patients with multiple myeloma. Here we show that HGF inhibited bone morphogenetic protein (BMP)-induced in vitro osteoblastogenesis. Thus, HGF in-**

**hibited BMP-induced expression of alkaline phosphatase in human mesenchymal stem cells (hMSCs) and the murine myoid cell line C2C12, as well as mineralization by hMSCs. Furthermore, the expression of the osteoblast-specific transcription factors Runx2 and Osterix was reduced by HGF treatment. HGF promoted proliferation of hMSCs, and the BMP-induced halt in proliferation was overridden by HGF, keeping the cells in a proliferative, undifferentiating state. BMP-induced nuclear translocation of receptor-activated**

**Smads was inhibited by HGF, providing a possible explanation of how HGF inhibits BMP signaling. The in vitro data were supported by the observation of a negative correlation between HGF and a marker of osteoblast activity, bone-specific alkaline phosphatase ( $\rho = -0.45$ ,  $P = .008$ ), in sera from 34 patients with myeloma. These observations suggest that HGF inhibits bone formation in multiple myeloma. (Blood. 2007;109:3024-3030)**

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## Introduction

Almost all patients with multiple myeloma develop osteolytic lesions and/or diffuse osteopenia. The presence of myeloma cells in the bone marrow leads to both increased number and activity of osteoclasts as well as reduced number and dysfunction of osteoblasts.<sup>1,2</sup> During the early stages of the disease, enhanced osteoclast activation is at least partly compensated by an increase in bone formation. As the disease progresses, the osteoblasts are fewer and less active, so that bone resorption is uncoupled from bone formation.<sup>3</sup> Recently, several factors have been proposed to be involved in the dysregulation of osteoblastogenesis in multiple myeloma.<sup>4-7</sup> However, the mechanisms for reduced bone formation in patients with myeloma are still not completely understood.

We have shown that myeloma cells produce hepatocyte growth factor (HGF).<sup>8</sup> Others have shown that HGF is among the most upregulated genes in myeloma cells as compared with normal plasma cells.<sup>9</sup> High serum concentration of HGF is associated with poor prognosis in patients with myeloma,<sup>10</sup> and HGF has been shown to play several roles in disease progression.<sup>11-16</sup> Furthermore, we have shown that HGF is found at high concentrations in bone marrow plasma from patients with myeloma.<sup>17</sup>

In a mouse model of multiple myeloma developed in our laboratory, a prominent feature was the complete lack of osteoblasts.<sup>18</sup> In this model, JJN3 cells, which produce high levels of HGF, were injected into severe combined immunodeficient (SCID) mice. The concentration of HGF in the bone marrow plasma of the mice was high; in some mice the concentration exceeded

5  $\mu\text{g/mL}$ .<sup>18</sup> We therefore hypothesized that HGF can interfere with osteoblastogenesis, and thereby be a factor responsible for the reduced number and reduced activity of osteoblasts in patients with multiple myeloma.

## Materials and methods

### Cells and cell culture conditions

C2C12 cells (ATTC, Rockville, MD) were routinely maintained in Dulbecco modified Eagle medium (DMEM; Gibco, Carlsbad, CA) with 15% heat inactivated fetal calf serum (FCS; EuroClone, Pero, Italy) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Cells were not allowed to reach confluence, and were used before they completed 8 passages. Human mesenchymal stem cells (hMSCs) were purchased from Cambrex Bio Science and routinely maintained in human mesenchymal stem cells growth media (MSCGM; Cambrex Bio Science, East Rutherford, NJ) according to the manufacturer's instructions. hMSCs were used before they completed 8 passages.

### Osteoblast differentiation

hMSCs were seeded at 4000 cells/cm<sup>2</sup> and cultured until they reached 70% confluence. Then, osteogenic differentiation was induced by adding dexamethasone (10<sup>-8</sup> M; Sigma-Aldrich, St Louis, MO), beta-glycerophosphate (10 mM; Sigma-Aldrich), and L-ascorbic acid (0.05 mM; Sigma-Aldrich) with or without BMP-2 (300 ng/mL; R&D Systems, Minneapolis, MN) and HGF (100 ng/mL; R&D Systems). Media were renewed every 3 to 4 days.

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C2C12 cells were seeded at 10 000 cells/cm<sup>2</sup> in DMEM/1% FCS with or without BMP-2 (300 ng/mL) and HGF (100 ng/mL).

### ALP assay

Alkaline phosphatase (ALP) activity was quantified by ELF 97 Endogenous Phosphatase Detection Kit (Molecular Probes, Eugene, OR). Briefly, cells in 96-well plates were fixed in 3% para-formaldehyde for 10 minutes at room temperature. To permeabilize the cells, 200  $\mu$ L/well of phosphate-buffered saline (PBS)/0.2% Tween-20 was added and the plates were incubated for 15 minutes. The cells were briefly rinsed twice in water, and then left for 10 minutes in H<sub>2</sub>O. Substrate was diluted 1:20 in substrate buffer, 50  $\mu$ L/well, and incubated for 5 minutes. Then, fluorescence was detected using a multilabel counter (Viktor 1420; Perkin Elmer, Wellesley, MA) with excitation filter at 355 nm and emission filter at 535 nm. To adjust for differences in cell number, cells were subsequently lysed in either 100  $\mu$ L 10% SDS or 150  $\mu$ L 1X SSC/100  $\mu$ g/mL proteinase K, and total protein (Micro BCA protein assay kit; Pierce, Rockford, IL) or DNA (SybrGreen), respectively, were measured. In parallel, to visualize ALP expression, cells were stained with the Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich) according to the manufacturer's instruction.

### Mineralization

hMSCs were seeded in 24-well plates and cultured for 14 and 17 days. Media were renewed every 3 to 4 days. Cultures were terminated by fixing cells in 3% para-formaldehyde for 10 minutes on ice. Fixed cultures were rinsed with PBS and then with water, thereafter stained with 500  $\mu$ L/well 40 mM Alizarin Red-S (ARS; Sigma-Aldrich) at pH 4.2 and room temperature with gentle agitation. Cells were then washed 5 times with water followed by a 15-minute rinse with PBS under gentle rotation. Stained cultures were photographed, then destained by incubating in 10% (wt/vol) cetylpyridinium chloride (CPC) in 10 mM sodium phosphate buffer pH 7.0 for 1 hour at room temperature. ARS concentration in these extracts was determined by absorbance at 562 nm. A standard curve was obtained by diluting ARS in CPC.

### Real-time RT-PCR

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Applied Science, Indianapolis, IN). A Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science) was used to synthesize cDNA using oligo dT-primers according to the manufacturer's instructions. RNA (2  $\mu$ g) was used as a template in the cDNA synthesis reaction. PCR was carried out with the LC Fast Start DNA Master SYBR Green kit (Roche Applied Science) using 2.0  $\mu$ L cDNA, corresponding to 20 ng total RNA in 20  $\mu$ L final volume with 4 mM MgCl<sub>2</sub> and 0.5  $\mu$ M of each primer. Relative quantitative PCR was performed using LightCycler (Roche Applied Science). Briefly, after an initial step of denaturation at 95°C for 20 seconds, 45 cycles of amplification were run containing the following steps: 95°C for 10 seconds, specific annealing temperature for 5 seconds, and extension at 72°C for the number of seconds as determined by the formula: product size/25. Amplification specificity was checked using melting curve analysis. Primers and annealing temperatures were as follows: *RUNX2*, forward 5'-CCAAGAAGGCACAGACAGAAGC-3' and reverse 5'-CTGCCTG-GCTCTTCTACTGAG-3', annealing temperature 68°C, extension time 17 seconds; *Osterix*, forward 5'-ATGGCGTCTCTCTGCTTGA-3' and reverse 5'-GGGAGCAAAGTCAGATGGGTAA-3', annealing temperature 64°C, extension time 27 seconds; *GAPDH*, forward 5'-CACCATG-GAGAAGGC-3' and reverse 5'-GACGGACACATTGGGGGTAG-3'. Results were analyzed with LightCycler Software v.3.5 (Roche Applied Science) using the second derivative maximum method to set crossing points. The crossing points obtained are a function of the amplification efficiency of the respective PCR. To adjust for differences in PCR efficiency, relative standard curves were obtained from 5-fold dilutions of pooled cDNA (corresponding to 40 ng RNA to 32 pg RNA) from BMP-2-treated C2C12 cells for both target and reference genes. Then, calibrator-normalized relative quantification with efficiency correction was carried out with Real Quant Software (Roche Applied Science). Samples

were run in triplicate. The ratio of target mRNA to GAPDH in control samples was set to 1.

### Western blot

Nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. Protein (30–100  $\mu$ g) was loaded onto precasted 10% to 12% Bis-Tris Gels (Invitrogen, Carlsbad, CA) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Gels were then blotted onto nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk in 0.1% Tween-20-Tris buffered saline pH 7.4 (TBST) for 1 hour at room temperature. Anti-Runx2 (clone 8G5; MBL International, Woburn, MA, or R&D Systems), anti-phospho-Smads 1, 5, and 8 (Cell Signaling, Danvers, MA), anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-GAPDH (AbCam) were incubated overnight at 4°C in 5% nonfat dry milk in TBST. Membranes were washed 4 times and incubated with an appropriate secondary antibody for 1 hour at room temperature. Following 4 washes in TBST the membranes were developed with enhanced chemiluminescence detection reagents (ECL; Amersham Biosciences, Amersham, United Kingdom) according to manufacturer's instructions and exposed to Hyperfilm ECL (Amersham Biosciences).

### Proliferation

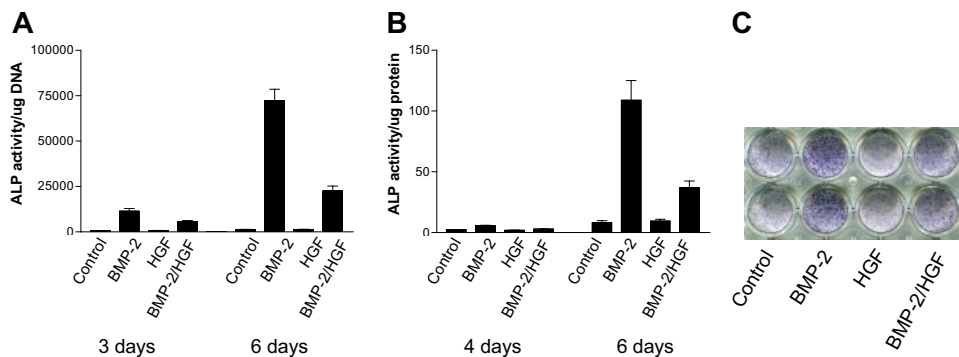
To measure DNA synthesis, hMSCs were seeded in 96-well plates at a density of 5000 cells/well in 200  $\mu$ L MSCGM supplemented with dexamethasone (10<sup>-8</sup> M), beta-glycerophosphate, L-ascorbic acid (0.05 mM), and cytokines as indicated for each experiment. After 1, 2, or 7 days, the cells were pulsed with 0.75  $\mu$ Ci (27/750 Bq) methyl-<sup>3</sup>H-thymidine (NEN Life Science Products, Boston, MA) per well and harvested 18 hours later with a Micromate 96-well harvester (Packard, Meriden, CT). Beta radiation was measured with a Matrix 96 counter (Packard).

### Immunofluorescence microscopy

C2C12 cells were serum starved for 24 hours before treatment with BMP-2 (300 ng/mL) and/or HGF (100 ng/mL) for 45 minutes. Then the cells were fixed on ice with 3% para-formaldehyde in PBS for 10 minutes. Subsequently, the cells were blocked and permeabilized with 2.5% BSA/20% A+serum/0.2% saponin in PBS (blocking/permeabilization buffer) for 20 minutes. Anti-phospho-Smads 1, 5, and 8 (Cell Signaling) were diluted 1:100 in blocking buffer and incubated for 1 hour. After 3 washes in blocking buffer, Alexa-633-conjugated goat antirabbit (Molecular Probes) was added and cells were incubated for 45 minutes. The cells were subsequently washed twice before examination. Images were acquired and viewed using an Axiovert 100M microscope equipped with a Zeiss LSM510 Meta scanning unit and a 63 $\times$ /1.4 numerical aperture oil immersion objective (all from Zeiss, Jena, Germany). Captured images were processed using Zeiss LSM510 software and Adobe Photoshop (Adobe Systems, San Jose, CA). Unless otherwise stated, the procedure was performed at room temperature. Cells that had fluorescence intensity in the nucleus higher than a certain threshold (100 pixels) were counted as positive for phospho-Smad 1, 5, and 8 nuclear translocation.

### Transfection and reporter assay

hMSCs (500 000) were transfected with 2.0  $\mu$ g of the BMP-responsive luciferase reporter construct BRE-Luc<sup>19</sup> (kindly provided by Dr O. Korchynskyi and Prof Peter ten Dijke) using a human MSC Nucleofector Kit (Amaxa Biosystems, Cologne, Germany) and program C-17 on the Amaxa Nucleofector (Amaxa Biosystems). The cells were then seeded in 96-well plates, 30 000 cells/well in MSCGM, and cultured overnight. The next day, cells were stimulated for 5 hours in MSCGM with or without BMP-2 (300 ng/mL) or HGF (100 ng/mL). Then media were removed, the cells washed with PBS, and 20  $\mu$ L 1X cell culture lysis reagent (Promega, Madison, WI) were added to lyse the cells. Luciferase assay substrate (70  $\mu$ L; Promega) were added to 15  $\mu$ L of the lysates, and luciferase activity was measured using a luminometer (Viktor 1420; Perkin Elmer). By transfecting hMSCs with pMaxGFP (Amaxa Biosystems) the transfection efficiency was determined to be 40% to 50%.



**Figure 1. HGF inhibits BMP-2-induced ALP activity in vitro.** ALP activity of C2C12 cells (A) and hMSCs (B-C). The hMSCs in (C) had been treated with cytokines for six days. The concentrations of cytokines were 300 ng/mL BMP-2 and 100 ng/mL HGF. Data are presented as means + SEM.

## Patients

Sera from 34 patients with newly diagnosed multiple myeloma were analyzed in the *in vivo* part of the study. All patients had symptomatic disease and an expected distribution of myeloma subtypes: 25 patients had IgG subtype, 5 patients IgA, 3 patients light chain disease, and 1 patient nonsecretory disease. All but 2 patients had lytic bone disease and/or pathologic fractures. Sera were taken before start of treatment and stored at  $-80^{\circ}\text{C}$  until analysis. The study was approved by the local ethics committee at Aarhus University Hospital. Informed consent was provided according to the Declaration of Helsinki. Patient characteristics have been described in detail elsewhere.<sup>20</sup>

## Serum assays

HGF in serum was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems).

**Markers of bone formation.** Total alkaline phosphatase activity in serum was measured spectrophotometrically using *p*-nitrophenylphosphate as a substrate, and the bone-specific alkaline phosphatase (bALP) was determined by lectin precipitation.<sup>21</sup> The N-terminal propeptide (PINP) and the C-terminal propeptide (PICP) of procollagen type I were measured by radioimmunoassay (RIA) from Farnos Diagnostica (Oulunsalo, Finland).<sup>22,23</sup> Osteocalcin (OCN) in serum was determined by RIA.<sup>24</sup>

**Markers of bone resorption.** Serum concentration of C-terminal telopeptide of collagen type I (ICTP) was analyzed by 2 different assays, the ICTP RIA assay from Farnos Diagnostica, which uses rabbit polyclonal antibodies for detection of a cross-linked fragment liberated by metalloproteinase activity,<sup>25,26</sup> and the Ctx-1  $\beta$ -Crosslaps electrochemoluminescence immunoassay from Roche Diagnostics (Mannheim, Germany), which uses 2 monoclonal murine antibodies recognizing  $\beta$ -8AA octapeptides within telopeptide fragments liberated by cathepsin K.<sup>27,28</sup> The N-terminal telopeptide of collagen type I (Ntx-1) in urine was measured by an ELISA inhibition assay from Ostex (Seattle, WA).<sup>29</sup> Ntx-1 results were expressed as nmol bone collagen equivalent per mmol creatinine.

## Statistics

Statistical analyses were performed with the SPSSX/PC computer program (SPSS, Chicago, IL). Results were considered statistically significant when  $P < .05$ . Skewed variables (Kurtosis  $> 7$ ) were transformed by the natural logarithm (ln) before entering analyses requiring normal distribution.

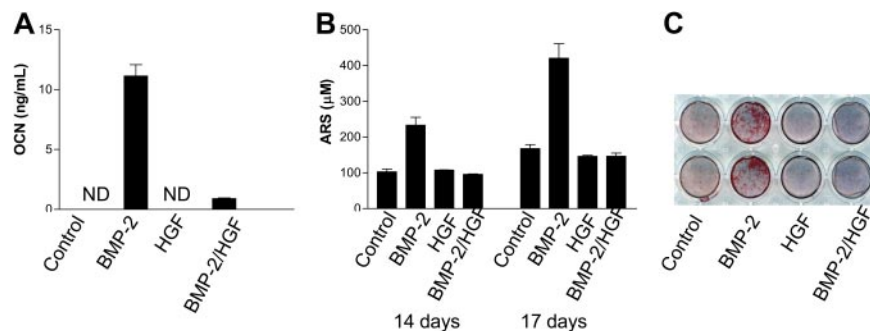
## Results

### HGF inhibits osteoblast differentiation in vitro

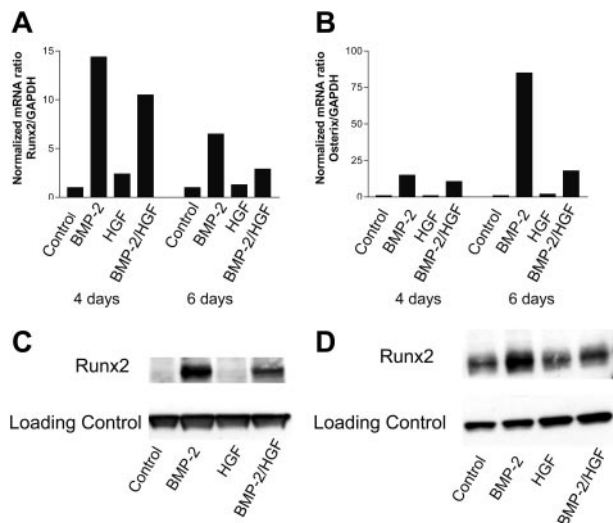
We investigated the role of HGF in BMP-induced osteoblastogenesis using 2 different *in vitro* model systems. C2C12 cells are murine myoid cells that differentiate to osteoblasts when they are treated with BMP-2.<sup>30</sup> Likewise, when hMSCs are cultured in osteogenic media (see "Materials and methods") osteoblast differentiation is induced, and the differentiation is enhanced when the cells are treated with BMP-2. ALP is frequently used as a marker for early osteoblast differentiation, whereas osteocalcin and the accumulation of calcium are used as markers for terminally differentiated osteoblasts.<sup>31</sup> ALP activity of both C2C12 cells (Figure 1A) and hMSCs (Figure 1B-C) was inhibited by the addition of C2C12 cells (Figure 2A), and mineralization of osteoblasts derived from hMSCs (Figure 2B-C) was inhibited by HGF. Thus, HGF inhibited both early and late stages of BMP-induced osteoblast differentiation.

### HGF inhibits the expression of osteoblast-specific transcription factors Runx2 and Osterix

Osteoblast differentiation is regulated by sequential expression of the osteoblast-specific transcription factors Runx2 and Osterix.<sup>32-35</sup> In C2C12 cells, *RUNX2* mRNA was expressed at high levels after 4 days of BMP-2 treatment, and although still present, the expression decreased after another 2 days of culturing (Figure 3A). Osterix, on the other hand, was induced later in the differentiation process and showed high expression after 6 days of BMP-2 treatment (Figure 3B). HGF inhibited the BMP-2-induced expression of both *RUNX2* and Osterix mRNA (Figure 3A-B). The decrease of BMP-2-induced *RUNX2* mRNA in C2C12 cells in the presence of HGF resulted in a decrease of Runx2 protein expression as shown in Figure 3C. hMSCs cultured in osteogenic media showed a basal Runx2 protein expression (Figure 3D). BMP-2 treatment



**Figure 2. HGF inhibits BMP-2-induced terminal differentiation of osteoblasts.** Osteocalcin concentration (A) in conditioned media from C2C12 cells cultured for 6 days. Mineralization of MSCs treated for the indicated time periods was quantified (B) or visualized (C) by Alizarin Red-S (ARS) staining. The concentrations of cytokines were 300 ng/mL BMP-2 and 100 ng/mL HGF. ND indicates not detected.

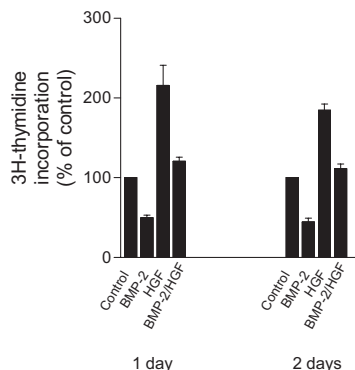


**Figure 3. HGF inhibits the BMP-2-induced expression of osteoblast-specific transcription factors.** Relative mRNA levels of Runx2 (A) and Osterix (B) in C2C12 cells. Runx2 protein expression detected by Western blotting of nuclear extracts from C2C12 cells (C) and hMSCs (D) treated for 6 days. The concentrations of cytokines were 300 ng/mL BMP-2 and 100 ng/mL HGF.

enhanced this basal expression, and more importantly, HGF inhibited the BMP-induced Runx2 protein expression also in hMSCs (Figure 3D). In conclusion, HGF inhibits the BMP-2-induced expression of the osteoblast-specific transcription factors Runx2 and Osterix.

**HGF promotes proliferation of human mesenchymal stem cells**

When mesenchymal stem cells differentiate, proliferation is inhibited.<sup>31</sup> In line with this, we found that BMP-2 inhibited the proliferation of hMSCs (Figure 4). Moreover, although HGF alone had no effect on the expression of osteoblast markers (Figures 1 and 2), we found that HGF treatment promoted proliferation of hMSCs as compared with both untreated and BMP-2-stimulated cells (Figure 4). Furthermore, HGF abolished the antiproliferative effect of BMP-2 (Figure 4), indicating that HGF keeps the cells in a proliferative, undifferentiated state. The influence on proliferation was most pronounced during the first 2 days after osteogenic induction; after 7 days, there was no significant difference in proliferation rates (data not shown). In contrast, neither BMP-2 nor HGF had any significant influence on proliferation of C2C12 cells (data not shown).



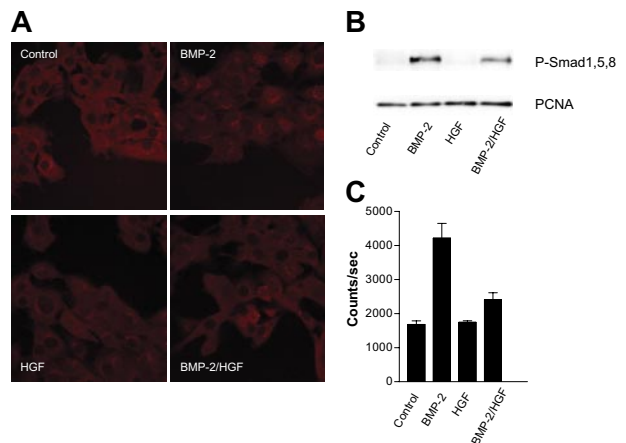
**Figure 4. HGF promotes proliferation of MSCs.** MSCs were treated for 1 or 2 days as indicated before <sup>3</sup>H-thymidine was added to the media. The amount of incorporated <sup>3</sup>H-thymidine was measured after another 18 hours of culturing. Bars represent mean + SEM of triplicate wells. Control levels of <sup>3</sup>H-thymidine incorporation per well were 3866 counts/min on day 1 and 2969 counts/min on day 2. The concentrations of cytokines were 300 ng/mL BMP-2 and 100 ng/mL HGF.

**HGF inhibits BMP-2 signaling**

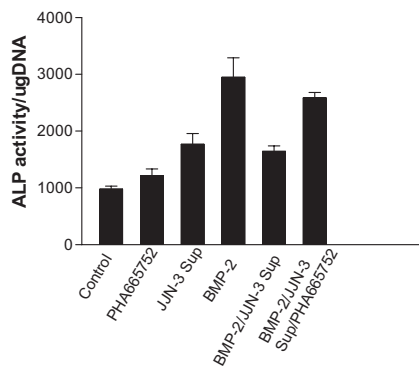
The osteogenic activity of BMPs is partly mediated by nuclear translocation of Smads,<sup>36</sup> so we wanted to investigate whether HGF acts on osteoblastogenesis by interfering with Smad signaling. By confocal microscopy, we looked at the presence of Smads 1, 5, and 8 in the nucleus of C2C12 cells treated with BMP-2 and/or HGF. As shown in Figure 5A, HGF treatment reduced the nuclear translocation of Smads 1, 5, and 8. In 57% of the cells treated with BMP-2, Smads 1, 5, and 8 were present in the nucleus, whereas in cells treated with both HGF and BMP-2 the presence of Smads 1, 5, and 8 was detected in only 21% of the nuclei, which was similar to the level in untreated cells (Figure 5A). We also performed Western blots of nuclear extracts of C2C12 cells and found a strong BMP-2-induced nuclear localization of Smads 1, 5, and 8. The BMP-2-induced nuclear translocation of Smads 1, 5, and 8 was reduced in the presence of HGF (Figure 5B). To investigate the effect of HGF on Smad signaling in hMSCs, we employed a Smad-driven BMP-reporter construct. As shown in Figure 5C, HGF inhibited Smad signaling in hMSCs, suggesting that HGF interferes with immediate BMP signaling also in hMSCs.

**The inhibitory effect of conditioned media from myeloma cells on BMP-2-induced osteoblastogenesis is abolished by inhibiting HGF signaling**

It is shown that patients with myeloma have elevated levels of HGF in the bone marrow.<sup>17</sup> To further verify that HGF derived from myeloma cells is involved in osteoblast inhibition we tested whether blocking of HGF signaling in hMSCs would abolish the inhibitory effect of conditioned media from the HGF-producing myeloma cell line JJN3. Although a 1:10 dilution of JJN3-conditioned media slightly increased the basal ALP activity of hMSCs, the BMP-2-induced ALP activity was inhibited (Figure 6). A small molecule ATP-competitive inhibitor of c-Met tyrosine kinase (PHA665 752)<sup>37</sup> almost completely reversed the inhibition of osteoblast differentiation, indicating that at least part of the inhibitory action of JJN3 supernatant on BMP-induced hMSC differentiation is due to the presence of HGF.



**Figure 5. HGF inhibits Smad signaling.** (A) Detection of phosphorylated (P)-Smad 1, 5, and 8 in C2C12 cells treated for 45 minutes with BMP-2 (300 ng/mL) and/or HGF (100 ng/mL). (i) Control cells (P-Smad 1, 5, or 8 detected in 19% of the nuclei); (ii) BMP-2-treated cells (P-Smad 1, 5, or 8 detected in 57% of the nuclei); (iii) HGF-treated cells (P-Smad 1, 5, or 8 detected in 33% of the nuclei); and (iv) BMP-2- and HGF-treated cells (P-Smad 1, 5, or 8 detected in 21% of the nuclei). (B) Detection of P-Smad 1, 5, and 8 by Western blotting of nuclear extracts of C2C12 cells treated for 45 minutes as indicated. (C) Luciferase activity of hMSCs transfected with BRE-luc stimulated for 5 hours as indicated. The concentrations of cytokines in (A-C) were 300 ng/mL BMP-2 and 100 ng/mL HGF.



**Figure 6. A c-met inhibitor (PHA665752) blunts the inhibitory effect of conditioned media from JJN cells on BMP-2-induced osteoblastogenesis.** ALP activity of hMSCs treated with PHA665752 (100 nM), conditioned media from JJN3 cells diluted 1:10, and BMP-2 (300 ng/mL) for 4 days as indicated. Differences in ALP activity were statistically significant (independent samples, 2-tailed t test) in untreated cells versus cells treated with JJN3 Sup ( $P = .007$ ), in untreated cells versus BMP-2-treated cells ( $P = .001$ ), in BMP-2-treated cells versus cells treated with BMP-2/JJN-3 Sup ( $P = .008$ ), and in BMP-2/JJN-3 Sup-treated cells versus cells treated with BMP-2/JJN-3 Sup/PHA665752.

### HGF correlates negatively with markers of osteoblast activity in patients with multiple myeloma

Since we found that HGF inhibited osteoblastogenesis in vitro, we wanted to investigate whether serum concentrations of HGF correlate with markers of osteoblast activity in patients with myeloma. We found that HGF correlated significantly to bone-specific alkaline phosphatase (bALP) in a Spearman rank correlation analysis ( $r = -0.45$ ,  $P = .008$ ; Table 1 and Figure S1, available on the *Blood* website; see the Supplemental Figure link at the top of the online article). Serum bALP is a specific marker of bone formation rate in patients with multiple myeloma,<sup>38</sup> so the finding of a negative correlation between bALP and HGF supports our in vitro data of HGF as an inhibitor of osteoblastogenesis. When we logarithmically transformed skewed variables before entering a Pearson correlation analysis, PINP ( $r = -0.374$ ,  $P = .029$ ) came out in addition to bALP ( $r = -0.516$ ,  $P = .002$ ) as negatively correlated to HGF. PINP is another marker for osteoblast activity. Taken together, this strengthens our hypothesis of HGF as an osteoblast-inhibiting factor in multiple myeloma.

## Discussion

BMP signaling has been shown to be essential to obtain osteoblasts in adult animals.<sup>39-41</sup> In this report, we show that HGF inhibits BMP-2-induced osteoblastogenesis in vitro. The expression of both early and late markers of osteoblastogenesis was inhibited by HGF treatment, including the osteoblast-specific transcription factors Runx2 and Osterix. Furthermore, in patients with myeloma, serum concentrations of HGF were negatively correlated to markers of osteoblast activity.

HGF is on the top 10 list of genes that distinguishes myeloma from Waldenström macroglobulinemia and chronic lymphocytic

leukemia,<sup>42</sup> both B-cell malignancies with some similarity to myeloma, but with little or no bone affection. On the same list, members of the Wnt-family Dickkopf-1 (Dkk1) and Frizzled-related protein are found. These known inhibitors of Wnt signaling have previously been suggested to be involved in osteoblast dysfunction in patients with myeloma.<sup>4,5</sup> Wnt signaling is important for normal skeletogenesis<sup>43</sup> and synergizes with BMP to promote osteoblast differentiation.<sup>44,45</sup> Thus, the myeloma cell may produce factors that inhibit both BMP signaling (HGF) and Wnt signaling (Dkk1 and sFRP2) pathways. Other factors reported to inhibit osteoblastogenesis in multiple myeloma are IL-7<sup>7</sup> and IL-3.<sup>6</sup> In contrast to HGF, IL-7 did not inhibit the expression of Runx2, although the transactivity of Runx2 was reduced.<sup>7</sup> IL-3 has been shown not to act directly on osteoblasts, but indirectly via CD45<sup>+</sup> cells by an unknown mechanism.<sup>6</sup>

We found that serum concentration of HGF in patients with myeloma correlated negatively with bALP. bALP is derived from osteoblast membranes and is a specific marker of osteoblast activity in patients with myeloma, correlating well with histomorphometrically estimated dynamic indices of bone formation.<sup>38</sup> HGF also correlated inversely with PINP, another marker of bone formation that reflects osteoblastic synthesis of collagen type I. Alexandrakis et al<sup>46</sup> found that HGF was negatively correlated to osteocalcin, and, although not significant, there was an inverse association of osteocalcin and HGF in our patient material as well. However, osteocalcin in the circulation may be both newly synthesized and released during resorption, so there is some question as to whether it should be considered a marker for osteoblast activity.<sup>47</sup> In addition, osteocalcin reflects relative osteoid surfaces and osteoid thickness more than dynamic indices of bone formation.<sup>38</sup> In a few clinical studies serum concentrations of HGF have been correlated to markers of osteoclast activity,<sup>10,46,48</sup> and recent data suggest that HGF may also directly stimulate osteoclastogenesis.<sup>49</sup> We did not find any significant correlations of HGF to osteoclast markers in our analyses. However, the number of patient samples available in this study was relatively small, which may explain our inability to find the significant correlations reported earlier.

Osteoblast differentiation is regulated by sequential expression of the osteoblast-specific transcription factors Runx2 and Osterix.<sup>32-35</sup> In line with previous studies,<sup>50-52</sup> we show that BMP-2 increases expression of Runx2, both in C2C12 cells as well as in hMSCs. HGF inhibits the BMP-2-induced expression of Runx2, but does not seem to inhibit the basal level of Runx2 present in hMSCs cultured in osteogenic media. Similarly, HGF inhibited BMP-2-induced ALP activity and mineralization, but not the basal ALP activity and mineralization present in hMSCs cultured in osteogenic media. However, it should be noted that in some experiments we observed an inhibition of the basal level of mineralization upon HGF treatment, but the reduction was nonsignificant, and might be due to the inhibition of autocrine BMP signaling.<sup>53</sup> HGF therefore seemed to specifically target BMP signaling pathways.

BMPs signal through activation of transmembrane serine/threonine kinase receptors. Ligand binding induces transphosphorylation of the type I receptor by the type II receptor kinase. The

**Table 1. Correlations between HGF and markers of bone resorption and bone formation in patients with myeloma, at diagnosis**

	ICTP	Ctx-1	Ntx-1	PINP	PICP	OCN	bALP
Spearman rho	0.214	0.037	-0.171	-0.316	-0.117	-0.149	-0.449*
Significance (2-tailed)	.159	.835	.333	.068	.443	.399	.008
N	45	34	34	34	45	34	34

\*Correlation is significant at the .01 level (2-tailed).

activated type I receptor then phosphorylates receptor Smads (R-Smads) at specific C-terminal serines. Together with Smad 4, the phosphorylated R-Smads translocate to the nucleus where they associate with other proteins to regulate transcription.<sup>36</sup> We found that HGF inhibited Smad signaling both in hMSCs and C2C12 cells, suggesting that the effect of HGF on BMP-induced osteoblastogenesis may be due to inhibition of important early steps in the BMP signaling pathway. Growth factors, including HGF, bind receptor tyrosine kinases that may activate the Ras-ERK pathway.<sup>54</sup> ERK has been shown to directly phosphorylate R-Smads at the linker region. This phosphorylation inhibits, by unknown mechanisms, the translocation of R-Smads to the nucleus both in vitro and in vivo,<sup>55,56</sup> and provides a possible explanation of how HGF inhibits Smad signaling. However, non-Smad pathways are also involved in BMP-induced osteoblast differentiation.<sup>57,58</sup> Furthermore, it has been shown that a Smad-independent RTK-Ras-ERK pathway inhibits BMP signaling.<sup>59</sup> Therefore, at this point we cannot exclude the possibility that HGF acts on other pathways in addition to the Smad pathway in the regulation of osteoblast differentiation.

The influence of growth factors on osteoblast differentiation is reported in several papers, although different mechanisms and even opposing effects of the same factors have been described.<sup>60-66</sup> It may be that transient exposure to mitotic growth factors such as FGF, EGF, and PDGF enhance osteogenesis, whereas continuous exposure inhibits osteoblast differentiation. The enhanced osteogenesis could be due to an increased self-renewal of mesenchymal stem cells that leads to an increase in the number of cells that can become fully mature osteoblasts when osteogenic stimuli are present. HGF has been shown by others to stimulate the expression of early osteoblast markers (ALP activity) in preosteoblasts,<sup>67</sup> and has also been reported to stimulate 1,25-dihydroxyvitamin D<sub>3</sub>-induced osteoblast differentiation by increased proliferation of osteoblast precursors.<sup>68</sup> In line with the latter study, we observed a proliferative effect of HGF on hMSCs. However, in contrast, HGF on its own had no effect on the differentiation toward osteoblasts, and, importantly, inhibited BMP-2-induced osteoblastogenesis. We propose that HGF may act on osteoblastogenesis in 2 different ways: by promoting self-renewal of hMSCs (possibly at the sacrifice of differentiation), and by interfering with BMP signaling, thus directly inhibiting BMP-induced osteoblastogenesis. In the bone marrow of patients with myeloma, the myeloma cells could

be a source of continuous production of HGF. Supporting this is the finding that myeloma cell-derived HGF has the same activity as recombinant HGF.

HGF is found at high concentrations in the bone marrow of patients with myeloma. We have previously shown that about 50% of the patients with multiple myeloma have elevated levels of HGF in bone marrow plasma when compared with healthy persons. In some of the patients the concentration of HGF in bone marrow plasma was extremely high, exceeding 100 ng/mL.<sup>17</sup> The differentiation of mesenchymal stem cells and osteoblast precursors in many patients with myeloma thus occurs in an environment containing abnormal levels of this cytokine. The in vitro and in vivo data presented in this study suggest that HGF reduces bone formation rates in patients with myeloma and hereby may contribute to the development of myeloma bone disease. Consequently, inhibition of HGF signaling could be useful in the treatment of the bone disease in multiple myeloma.

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## Authorship

Contribution: T.S. designed and performed research, analyzed data, and wrote the paper; N.A. collected data and performed research; U.M.F. and B.S. performed research; Ø.H. and M.B. designed research; and A.S. designed research and analyzed data.

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Correspondence: Therese Standal, Department of Cancer Research and Molecular Medicine, Olav Kyrresgt 5, MTFS, 3 etg N, N-7489 Trondheim, Norway; e-mail: therese.standal@ntnu.no.

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