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

Therese Standal,[1] Niels Abildgaard,[2] Unn-Merete Fagerli,[1] Berit Stordal,[1] Øyvind Hjertner,[1,3] Magne Borset,[1,4] and Anders Sundan[1]

[1]Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, Trondheim, Norway; [2]Department of Haematology, Odense University Hospital, Denmark; [3]Department of Hematology and [4]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 inhibited 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)

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.[1,2] 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.[3] Recently, several factors have been proposed to be involved in the dysregulation of osteoblastogenesis in multiple myeloma.[4-7] 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).[8] Others have shown that HGF is among the most upregulated genes in myeloma cells as compared with normal plasma cells.[9] High serum concentration of HGF is associated with poor prognosis in patients with myeloma,[10] and HGF has been shown to play several roles in disease progression.[11-16] Furthermore, we have shown that HGF is found at high concentrations in bone marrow plasma from patients with myeloma.[17]

In a mouse model of multiple myeloma developed in our laboratory, a prominent feature was the complete lack of osteoblasts.[18] 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 μg/mL.[18] 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_2 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^2 and cultured until they reached 70% confluence. Then, osteogenic differentiation was induced by adding dexamethasone (10^{-8} 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.

Submitted July 14, 2006; accepted November 18, 2006. Prepublished online as *Blood* First Edition Paper, November 30, 2006; DOI 10.1182/blood-2006-07-034884.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

C2C12 cells were seeded at 10 000 cells/cm^2 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 μL/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_2O. Substrate was diluted 1:20 in substrate buffer, 50 μL/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 μL 10% SDS or 150 μL 1X SSC/100 μg/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 μL/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 μg) 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 μL cDNA, corresponding to 20 ng total RNA in 20 μL final volume with 4 mM $MgCl_2$ and 0.5 μM 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′-CTGCCTGGCTCTTCTTACTGAG-3′, annealing temperature 68°C, extension time 17 seconds; Osterix, forward 5′-ATGGCGTCCTCTCTGCTTGA-3′ and reverse 5′-GGGAGCAAAGTCAGATGGGTAA-3′, annealing temperature 64°C, extension time 27 seconds; *GAPDH,* forward 5′-CACCATGGAGAAGGC-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 μg) 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 Bisociences).

Proliferation

To measure DNA synthesis, hMSCs were seeded in 96-well plates at a density of 5000 cells/well in 200 μL MSCGM supplemented with dexamethasone (10^{-8} 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 μCi (27/750 Bq) methyl-^{3}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×/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 μg of the BMP-responsive luciferase reporter construct BRE-Luc[19] (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 μL 1X cell culture lysis reagent (Promega, Madison, WI) were added to lyse the cells. Luciferase assay substrate (70 μL; Promega) were added to 15 μL 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%.

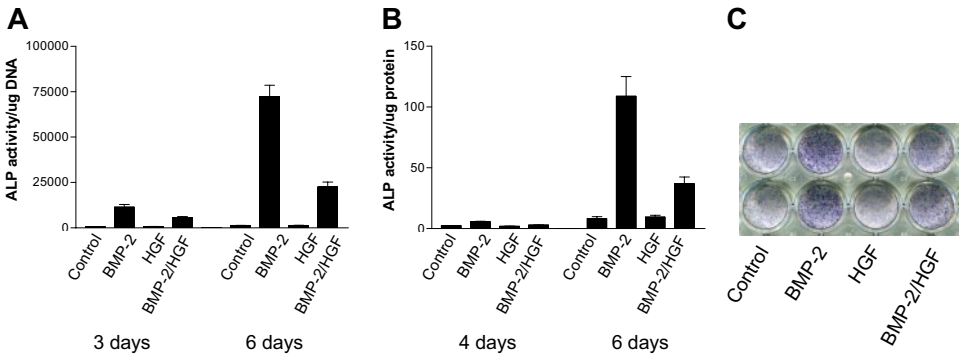

Figure 1. HGF inhibits BMP-2–induced ALP activity in vitro. ALP activity of C2C12 cells (A) and hMSCs (B-C). The hMSCs in (C) had been treated with cytokines for six days. The concentrations of cytokines were 300 ng/mL BMP-2 and 100 ng/mL HGF. Data are presented as means + SEM.

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°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.[20]

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.[21] The N-terminal propeptide (PINP) and the C-terminal propeptide (PICP) of procollagen type I were measured by radioimmunoassay (RIA) from Farmos Diagnostica (Oulunsalo, Finland).[22,23] Osteocalcin (OCN) in serum was determined by RIA.[24]

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 Farmos Diagnostica, which uses rabbit polyclonal antibodies for detection of a cross-linked fragment liberated by metalloproteinase activity,[25,26] and the Ctx-1 β-Crosslaps electrochemoluminescence immunoassay from Roche Diagnostics (Mannheim, Germany), which uses 2 monoclonal murine antibodies recognizing β-8AA octapeptides within telopeptide fragments liberated by cathepsin K.[27,28] The N-terminal telopeptide of collagen type I (Ntx-1) in urine was measured by an ELISA inhibition assay from Ostex (Seattle, WA).[29] 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.[30] 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.[31] ALP activity of both C2C12 cells (Figure 1A) and hMSCs (Figure 1B-C) was inhibited by the addition of HGF. Furthermore, BMP-2 induced osteocalcin expression 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.[32-35] 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

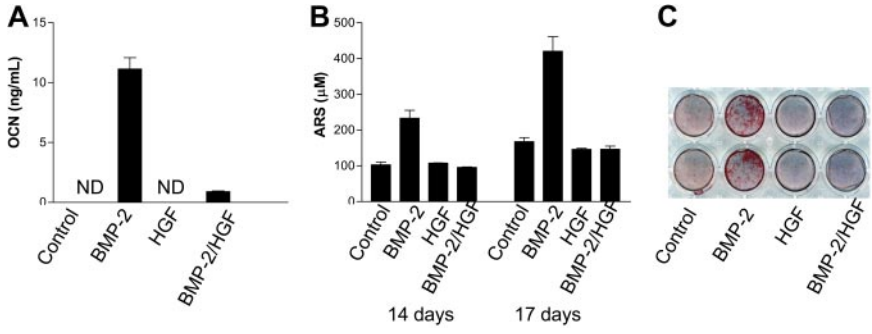

Figure 2. HGF inhibits BMP-2–induced terminal differentiation of osteoblasts. Osteocalcin concentration (A) in conditioned media from C2C12 cells cultured for 6 days. Mineralization of MSCs treated for the indicated time periods was quantified (B) or visualized (C) by Alizarin Red-S (ARS) staining. The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

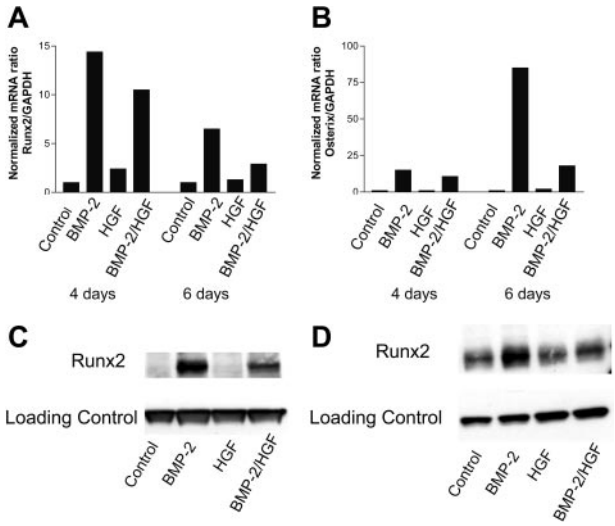

Figure 3. HGF inhibits the BMP-2–induced expression of osteoblast-specific transcription factors. Relative mRNA levels of Runx2 (A) and Osterix (B) in C2C12 cells. Runx2 protein expression detected by Western blotting of nuclear extracts from C2C12 cells (C) and hMSCs (D) treated for 6 days. The concentrations 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.[31] 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).

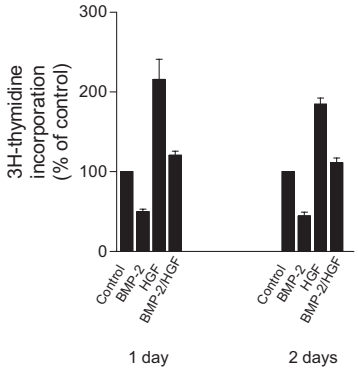

Figure 4. HGF promotes proliferation of MSCs. MSCs were treated for 1 or 2 days as indicated before ^{3}H-thymidine was added to the media. The amount of incorporated ^{3}H-thymdine was measured after another 18 hours of culturing. Bars represent mean + SEM of triplicate wells. Control levels of ^{3}H-thymidine 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,[36] 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.[17] 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)[37] 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.

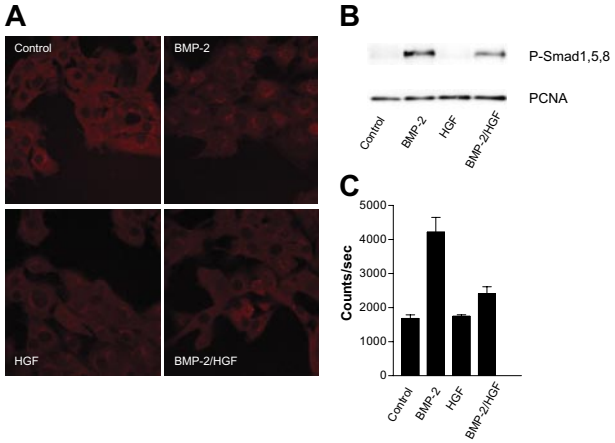

Figure 5. HGF inhibits Smad signaling. (A) Detection of phosphorylated (P)–Smad 1, 5, and 8 in C2C12 cells treated for 45 minutes with BMP-2 (300 ng/mL) and/or HGF (100 ng/mL). (i) Control cells (P–Smad 1, 5, or 8 detected in 19% of the nuclei); (ii) BMP-2–treated cells (P–Smad 1, 5, or 8 detected in 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.

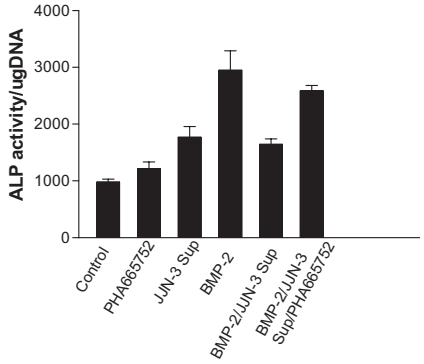

Figure 6. A c-met inhibitor (PHA665752) blunts the inhibitory effect of conditioned media from JJN cells on BMP-2–induced osteoblastogenesis. ALP activity of hMSCs treated with PHA665752 (100 nM), conditioned media from JJN3 cells diluted 1:10, and BMP-2 (300 ng/mL) for 4 days as indicated. Differences 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,[38] 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.[39-41] 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,[42] 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.[4,5] Wnt signaling is important for normal skeletogenesis[43] and synergizes with BMP to promote osteoblast differentiation.[44,45] 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[7] and IL-3.[6] In contrast to HGF, IL-7 did not inhibit the expression of Runx2, although the transactivity of Runx2 was reduced.[7] IL-3 has been shown not to act directly on osteoblasts, but indirectly via $CD45^+$ cells by an unknown mechanism.[6]

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.[38] HGF also correlated inversely with PINP, another marker of bone formation that reflects osteoblastic synthesis of collagen type I. Alexandrakis et al[46] 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.[47] In addition, osteocalcin reflects relative osteoid surfaces and osteoid thickness more than dynamic indices of bone formation.[38] In a few clinical studies serum concentrations of HGF have been correlated to markers of osteoclast activity,[10,46,48] and recent data suggest that HGF may also directly stimulate osteoclastogenesis.[49] 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.[32-35] In line with previous studies,[50-52] 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.[53] 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.[36] 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.[54] 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,[55,56] and provides a possible explanation of how HGF inhibits Smad signaling. However, non-Smad pathways are also involved in BMP-induced osteoblast differentiation.[57,58] Furthermore, it has been shown that a Smad-independent RTK-Ras-ERK pathway inhibits BMP signaling.[59] 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.[60-66] 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,[67] and has also been reported to stimulate 1,25-dihydroxyvitamin D_3–induced osteoblast differentiation by increased proliferation of osteoblast precursors.[68] 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.[17] 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.

Acknowledgments

We thank Dr Oleg Korchynskyi and Prof Peter ten Dijke for providing the BRE-luc construct, and Hanne Hella and Toril Holien for excellent technical assistance.

This work was supported by grants from the Norwegian Cancer Society, the Norwegian Research Council, the Cancer Fund, St Olav's Hospital, and the Grieg Foundation.

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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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.

References

1. Taube T, Beneton MN, McCloskey EV, et al. Abnormal bone remodelling in patients with myelomatosis and normal biochemical indices of bone resorption. Eur J Haematol. 1992;49:192-198.
2. Bataille R, Chappard D, Marcelli C, et al. Mechanisms of bone destruction in multiple myeloma: the importance of an unbalanced process in determining the severity of lytic bone disease. J Clin Oncol. 1989;7:1909-1914.
3. Bataille R, Chappard D, Marcelli C, et al. Recruitment of new osteoblasts and osteoclasts is the earliest critical event in the pathogenesis of human multiple myeloma. J Clin Invest. 1991;88:62-66.
4. Tian E, Zhan F, Walker R, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med. 2003;349:2483-2494.
5. Oshima T, Abe M, Asano J, et al. Myeloma cells suppress bone formation by secreting a soluble Wnt inhibitor, sFRP-2. Blood. 2005;106:3160-3165.
6. Ehrlich LA, Chung HY, Ghobrial I, et al. IL-3 is a potential inhibitor of osteoblast differentiation in multiple myeloma. Blood. 2005;106:1407-1414.
7. Giuliani N, Colla S, Morandi F et al. Myeloma cells block RUNX2/CBFA1 activity in human bone marrow osteoblast progenitors and inhibit osteoblast formation and differentiation. Blood. 2005; 106:2472-2483.
8. Borset M, Hjorth-Hansen H, Seidel C, Sundan A, Waage A. Hepatocyte growth factor and its receptor c-met in multiple myeloma. Blood. 1996;88: 3998-4004.
9. Zhan F, Hardin J, Kordsmeier B, et al. Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells. Blood. 2002;99:1745-1757.
10. Seidel C, Borset M, Turesson I, et al. Elevated serum concentrations of hepatocyte growth factor in patients with multiple myeloma. The Nordic Myeloma Study Group. Blood. 1998;91:806-812.
11. Andersen NF, Standal T, Nielsen JL, et al. Syndecan-1 and angiogenic cytokines in multiple myeloma: correlation with bone marrow angiogenesis and survival. Br J Haematol. 2005;128:210-217.
12. Hov H, Holt RU, Ro TB, et al. A selective c-met inhibitor blocks an autocrine hepatocyte growth factor growth loop in ANBL-6 cells and prevents migration and adhesion of myeloma cells. Clin Cancer Res. 2004;10:6686-6694.
13. Holt RU, Baykov V, Ro TB, et al. Human myeloma cells adhere to fibronectin in response to hepatocyte growth factor. Haematologica. 2005; 90:479-488.
14. Derksen PW, Keehnen RM, Evers LM, et al. Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. Blood. 2002;99: 1405-1410.
15. Hjertner O, Torgersen ML, Seidel C, et al. Hepatocyte growth factor (HGF) induces interleukin-11 secretion from osteoblasts: a possible role for HGF in myeloma-associated osteolytic bone disease. Blood. 1999;94:3883-3888.
16. Vande Broek I, Asosingh K, Allegaert V, et al. Bone marrow endothelial cells increase the invasiveness of human multiple myeloma cells through upregulation of MMP-9: evidence for a role of hepatocyte growth factor. Leukemia. 2004; 18:976-982.
17. Seidel C, Borset M, Hjertner O, et al. High levels of soluble syndecan-1 in myeloma-derived bone marrow: modulation of hepatocyte growth factor activity. Blood. 2000;96:3139-3146.

18. Hjorth-Hansen H, Seifert MF, Borset M, et al. Marked osteoblastopenia and reduced bone formation in a model of multiple myeloma bone disease in severe combined immunodeficiency mice. J Bone Miner Res. 1999;14:256-263.
19. Korchynskyi O, ten Dijke P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem. 2002;277:4883-4891.
20. Abildgaard N, Brixen K, Kristensen JE, et al. Comparison of five biochemical markers of bone resorption in multiple myeloma: elevated pretreatment levels of S-ICTP and U-Ntx are predictive for early progression of the bone disease during standard chemotherapy. Br J Haematol. 2003; 120:235-242.
21. Behr W, Barnert J. Quantification of bone alkaline phosphatase in serum by precipitation with wheat-germ lectin: a simplified method and its clinical plausibility. Clin Chem. 1986;32:1960-1966.
22. Melkko J, Kauppila S, Niemi S, et al. Immunoassay for intact amino-terminal propeptide of human type I procollagen. Clin Chem. 1996;42:947-954.
23. Melkko J, Niemi S, Risteli L, Risteli J. Radioimmunoassay of the carboxyterminal propeptide of human type I procollagen. Clin Chem. 1990;36: 1328-1332.
24. Price PA, Nishimoto SK. Radioimmunoassay for the vitamin K-dependent protein of bone and its discovery in plasma. Proc Natl Acad Sci U S A. 1980;77:2234-2238.
25. Risteli J, Elomaa I, Niemi S, Novamo A, Risteli L. Radioimmunoassay for the pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen: a new serum marker of bone collagen degradation. Clin Chem. 1993;39:635-640.
26. Sassi ML, Eriksen H, Risteli L, et al. Immunochemical characterization of assay for carboxy-terminal telopeptide of human type I collagen: loss of antigenicity by treatment with cathepsin K. Bone. 2000;26:367-373.
27. Rosenquist C, Fledelius C, Christgau S, et al. Serum CrossLaps One Step ELISA: first application of monoclonal antibodies for measurement in serum of bone-related degradation products from C-terminal telopeptides of type I collagen. Clin Chem. 1998;44:2281-2289.
28. Garnero P, Delmas PD. An immunoassay for type I collagen alpha 1 helicoidal peptide 620-633, a new marker of bone resorption in osteoporosis. Bone. 2003;32:20-26.
29. Hanson DA, Eyre DR. Molecular site specificity of pyridinoline and pyrrole cross-links in type I collagen of human bone. J Biol Chem. 1996;271: 26508-26516.
30. Katagiri T, Yamaguchi A, Komaki M, et al. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol. 1994;127:1755-1766.
31. Stein GS, Lian JB, van Wijnen AJ, et al. Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression. Oncogene. 2004;23:4315-4329.
32. Komori T, Yagi H, Nomura S, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell. 1997;89:755-764.
33. Otto F, Thornell AP, Crompton T, et al. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell. 1997;89:765-771.
34. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell. 1997;89:747-754.
35. Nakashima K, Zhou X, Kunkel G, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell. 2002;108:17-29.
36. ten Dijke P, Fu J, Schaap P, Roelen BA. Signal transduction of bone morphogenetic proteins in osteoblast differentiation. J Bone Joint Surg Am. 2003;85-A(suppl 3):34-38.
37. Christensen JG, Schreck R, Burrows J, et al. A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes in vitro and exhibits cytoreductive antitumor activity in vivo. Cancer Res. 2003;63:7345-7355.
38. Abildgaard N, Glerup H, Rungby J, et al. Biochemical markers of bone metabolism reflect osteoclastic and osteoblastic activity in multiple myeloma. Eur J Haematol. 2000;64:121-129.
39. Abe E, Yamamoto M, Taguchi Y, et al. Essential requirement of BMPs-2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by noggin. J Bone Miner Res. 2000;15:663-673.
40. Mishina Y, Starbuck MW, Gentile MA, et al. Bone morphogenetic protein type IA receptor signaling regulates postnatal osteoblast function and bone remodeling. J Biol Chem. 2004;279:27560-27566.
41. Zhao M, Harris SE, Horn D, et al. Bone morphogenetic protein receptor signaling is necessary for normal murine postnatal bone formation. J Cell Biol. 2002;157:1049-1060.
42. Chng WJ, Schop R, Price-Troska T, et al. Gene expression profiling of Waldenstrom's macroglobulinemia reveals a phenotype more similar to chronic lymphocytic leukemia than multiple myeloma. Blood. 2006;108:2755-2763.
43. Westendorf JJ, Kahler RA, Schroeder TM. Wnt signaling in osteoblasts and bone diseases. Gene. 2004;341:19-39.
44. Mbalaviele G, Sheikh S, Stains JP, et al. Beta-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation. J Cell Biochem. 2005;94:403-418.
45. Rawadi G, Vayssiere B, Dunn F, Baron R, Roman-Roman S. BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J Bone Miner Res. 2003; 18:1842-1853.
46. Alexandrakis MG, Passam FH, Sfiridaki A, et al. Elevated serum concentration of hepatocyte growth factor in patients with multiple myeloma: correlation with markers of disease activity. Am J Hematol. 2003;72:229-233.
47. Christenson RH. Biochemical markers of bone metabolism: an overview. Clin Biochem. 1997;30: 573-593.
48. Iwasaki T, Hamano T, Ogata A, et al. Clinical significance of vascular endothelial growth factor and hepatocyte growth factor in multiple myeloma. Br J Haematol. 2002;116:796-802.
49. Adamopoulos IE, Xia Z, Lau YS, Athanasou NA. Hepatocyte growth factor can substitute for M-CSF to support osteoclastogenesis. Biochem Biophys Res Commun. 2006;350:478-483.
50. Viereck V, Siggelkow H, Tauber S, et al. Differential regulation of Cbfa1/Runx2 and osteocalcin gene expression by vitamin-D3, dexamethasone, and local growth factors in primary human osteoblasts. J Cell Biochem. 2002;86:348-356.
51. Lee KS, Hong SH, Bae SC. Both the Smad and p38 MAPK pathways play a crucial role in Runx2 expression following induction by transforming growth factor-beta and bone morphogenetic protein. Oncogene. 2002;21:7156-7163.
52. Lee MH, Kim YJ, Kim HJ, et al. BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. J Biol Chem. 2003;278:34387-34394.
53. van der Horst G, van Bezooijen RL, Deckers MM, et al. Differentiation of murine preosteoblastic KS483 cells depends on autocrine bone morphogenetic protein signaling during all phases of osteoblast formation. Bone. 2002;31:661-669.
54. Schlessinger J. Common and distinct elements in cellular signaling via EGF and FGF receptors. Science. 2004;306:1506-1507.
55. Kretzschmar M, Doody J, Massague J. Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. Nature. 1997;389:618-622.
56. Pera EM, Ikeda A, Eivers E, De Robertis EM. Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. Genes Dev. 2003;17:3023-3028.
57. Nohe A, Hassel S, Ehrlich M, et al. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J Biol Chem. 2002;277:5330-5338.
58. Guicheux J, Lemonnier J, Ghayor C, et al. Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. J Bone Miner Res. 2003;18:2060-2068.
59. Nakayama K, Tamura Y, Suzawa M, et al. Receptor tyrosine kinases inhibit bone morphogenetic protein-Smad responsive promoter activity and differentiation of murine MC3T3-E1 osteoblast-like cells. J Bone Miner Res. 2003;18:827-835.
60. Fakhry A, Ratisoontorn C, Vedhachalam C, et al. Effects of FGF-2/-9 in calvarial bone cell cultures: differentiation stage-dependent mitogenic effect, inverse regulation of BMP-2 and noggin, and enhancement of osteogenic potential. Bone 2005; 36:254-266.
61. Sobue T, Naganawa T, Xiao L, et al. Over-expression of fibroblast growth factor-2 causes defective bone mineralization and osteopenia in transgenic mice. J Cell Biochem. 2005;95:83-94.
62. Mansukhani A, Ambrosetti D, Holmes G, Cornivelli L, Basilico C. Sox2 induction by FGF and FGFR2 activating mutations inhibits Wnt signaling and osteoblast differentiation. J Cell Biol. 2005;168:1065-1076.
63. Hurley MM, Abreu C, Harrison JR, et al. Basic fibroblast growth factor inhibits type I collagen gene expression in osteoblastic MC3T3-E1 cells. J Biol Chem. 1993;268:5588-5593.
64. Kubota K, Sakikawa C, Katsumata M, Nakamura T, Wakabayashi K. Platelet-derived growth factor BB secreted from osteoclasts acts as an osteoblastogenesis inhibitory factor. J Bone Miner Res. 2002;17:257-265.
65. Montero A, Okada Y, Tomita M, et al. Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. J Clin Invest. 2000;105:1085-1093.
66. Higuchi C, Myoui A, Hashimoto N, et al. Continuous inhibition of MAPK signaling promotes the early osteoblastic differentiation and mineralization of the extracellular matrix. J Bone Miner Res. 2002;17:1785-1794.
67. Hossain M, Irwin R, Baumann MJ, McCabe LR. Hepatocyte growth factor (HGF) adsorption kinetics and enhancement of osteoblast differentiation on hydroxyapatite surfaces. Biomaterials. 2005; 26:2595-2602.
68. D'Ippolito G, Schiller PC, Perez-stable C, et al. Cooperative actions of hepatocyte growth factor and 1,25-dihydroxyvitamin D3 in osteoblastic differentiation of human vertebral bone marrow stromal cells. Bone. 2002;31:269-275.

2007 109: 3024-3030
doi:10.1182/blood-2006-07-034884 originally published online November 30, 2006

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

Therese Standal, Niels Abildgaard, Unn-Merete Fagerli, Berit Stordal, Øyvind Hjertner, Magne Borset and Anders Sundan

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.