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Coordinated Regulation of Mesenchymal Stem Cell Differentiation on Microstructured Titanium Surfaces by Endogenous Bone Morphogenetic Proteins

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Abstract

Human mesenchymal stem cells (MSCs) differentiate into osteoblasts on microstructured titanium (Ti) surfaces without addition of medium supplements, suggesting that surface-dependent endogenous mechanisms are involved. They produce bone morphogenetic proteins (BMPs), which regulate MSC differentiation and bone formation via autocrine/paracrine mechanisms that are modulated by changes in BMP mRNA and protein, receptors, and inhibitors (Noggin, Cerberus, Gremlin 1, and Chordin). We examined expression of BMPs, their receptors and their inhibitors over time and used BMP2-silenced cells to determine how modulating endogenous BMP signaling can affect the process. MSCs were cultured on tissue culture polystyrene or Ti [PT (Ra<0.4μm); sandblasted/acid-etched Ti (SLA, Ra=3.2μm); or hydrophilic-SLA (modSLA)]. BMP mRNAs and proteins increased by day 4 of culture. Exogenous BMP2 increased differentiation whereas differentiation was decreased in BMP2-silenced cells. Noggin was regulated by day 2 whereas Gremlin 1 and Cerberus were regulated after 6 days. Osteoblastic differentiation increased in cells cultured with blocking antibodies against Noggin, Gremlin 1, and Cerberus. Endogenous BMPs enhance an osteogenic microenvironment whereas exogenous BMPs are inhibitory. Antibody blocking of the BMP2 inhibitor Cerberus resulted in IL-6 and IL-8 levels that were similar to those observed when treating cells with exogenous BMP2, while antibodies targeting the inhibitors Gremlin or Noggin did not. These results suggest that microstructured titanium implants supporting therapeutic stem cells may be treated with appropriately selected agents antagonistic to

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extracellular BMP inhibitors in order to enhance BMP2 mediated bone repair while avoiding undesirable inflammatory side effects observed with exogenous BMP2 treatment.

Keywords

Stromal/Stem Cells; BMP signaling; Titanium; Implants; Surface roughness

1. INTRODUCTION

Bone formation, healing, and regeneration are complex processes orchestrated by osteoprogenitor cells, osteoblasts, osteocytes, and osteoclasts. This requires a broad array of molecules that regulate cell differentiation, extracellular matrix synthesis, mineral deposition, and bone formation as well as bone remodeling. Several molecules influence osteoblastic differentiation *in vitro* and bone formation *in vivo* in both preclinical models and clinically [1]. Among these molecules, bone morphogenetic proteins 2, 4, and 7 (BMP2, BMP4, BMP7) are osteoinductive [2].

BMPs orchestrate several cellular functions such as cell proliferation, differentiation, transformation, and apoptosis in embryonic and adult cells via autocrine/paracrine mechanisms [2]. A complex system exists to ensure that these pleiotropic actions are regulated tightly both spatially and temporally. BMP molecules must dimerize to signal, either as heterodimers or as homodimers [3]. BMP receptors are heterodimers [2], providing additional levels of control with respect to availability of functional subunits. In addition, a number of intracellular proteins transduce the signal to the nucleus [3] and the relative availability of each of these may help modulate the rate and extent of the signaling cascade. Soluble inhibitors such as Noggin, Gremlin1, Cerberus, and Chordin physically bind to BMPs thereby regulating BMP signaling by preventing their dimerization and transduction of downstream events [4].

Recombinant human BMP2 is used clinically to induce bone formation in hard-to-heal fractures and bony defects in dental and orthopaedic applications [5,6]. Although it has been demonstrated to increase osteogenesis in lumbar spine fusions [7,8], inflammatory complications including seroma and osteolysis have been reported [9–12]. Titanium (Ti) dental implants that were dip-coated with BMP2 at concentrations typically used spine fusions also caused bone resorption in the mandible [10]. There are a number of possible reasons for this, but stimulatory effects of BMP2 on production of pro-inflammatory mediators may be a contributing factor.

A series of *in vitro* studies support this hypothesis. Osteoblasts cultured on microtextured Ti substrates produced higher levels of anti-inflammatory cytokines and lower levels of pro-inflammatory cytokines than cells on smooth Ti surfaces [13]. In addition, osteoblasts cultured on microstructured Ti or Ti alloy surfaces produced higher levels of BMP2, BMP4, and BMP7 [14,15], suggesting that osteoblast differentiation on the microtextured surfaces was due to intrinsic production of these osteoinductive proteins. Importantly, production of BMP inhibitors also increased on the microtextured substrates, providing a mechanism for regulating their paracrine action. However, when osteoblasts on microtextured Ti surfaces

were treated with exogenous BMP2, production of pro-inflammatory cytokines increased and production of anti-inflammatory cytokines decreased [13]. These results indicate that the intrinsic regulation of endogenous BMP2 signaling afforded by BMP2 inhibitors was insufficient to modulate the inflammatory effects of exogenous BMP2. These same microtextured Ti surface features result in reduced healing time and improved bone-to-implant contact clinically [16], suggesting that modifying paracrine BMP signaling may yield more robust bone formation than application of exogenous BMPs. In vivo studies using RNA interference to knockdown the BMP antagonist Noggin [17] showed enhanced bone formation, supporting this hypothesis. Inhibition of endogenous Noggin enhanced osteoblast maturation on microtextured Ti surfaces in vitro [15] and the BMP2 antagonist inhibitor L519 enhanced the osteogenic potential of BMP2 [17]. Thus, modifying paracrine BMP signaling may yield more robust bone formation than application of exogenous BMPs.

The stimulatory effects of microtexture on osteoblast differentiation are observed in cultures of human mesenchymal stem cells (MSCs), even in the absence of exogenous factors or osteogenic media, and this effect of surface microtopography is enhanced on rough hydrophilic Ti surfaces [18]. This raises the question of whether substrate-dependent endogenous BMP signaling is involved. Moreover, knockdown of Chordin has been shown to enhance osteogenic differentiation of MSCs on TCPS [19], suggesting that the effects of Ti surface microtopography on multipotent osteoprogenitor cells may also be modulated by manipulating levels of BMP inhibitors.

The purpose of this study was to examine the role of endogenous BMP in the regulation of peri-implant bone formation by assessing the expression and production of proteins involved in regulation of BMP action in osteoblastic differentiation of human MSCs cultured on microstructured Ti substrates. MSCs were cultured on Ti surfaces with two different surface topographies: a relatively smooth surface (PT) and a complex grit blasted and acid etched (SLA) surface. In addition, the role of surface chemistry was examined by culturing MSCs on Ti with a topography identical to the grit blasted/acid etched surface but with a hydrophilic chemistry (modSLA). Expression and protein levels for a number of proteins involved in BMP signaling, including BMPs and BMP receptor subunits and inhibitors, were determined, as were markers of osteoblastic differentiation, modulators of bone remodeling and angiogenesis, and inflammatory mediators. To assess the specific role of BMP2, MSCs stably silenced for BMP2 were generated. Finally, to determine whether inhibition of BMP2 action is involved in modulating BMP2 action, cells were treated with exogenous Noggin in addition to blocking the effects of BMP inhibitors using specific antibodies.

2. MATERIALS AND METHODS

2.1 Cell Culture

Human bone marrow-derived MSCs (Lonza Biosciences, Walkersville, MD) plated at a density of 10,000 cells per cm² were cultured in Mesenchymal Stem Cell Growth Media (MSCGM, Lonza Biosciences) at 37°C, 5% CO₂ and 100% humidity for all experiments.

2.2 Titanium Disks

Ti disks were prepared from 1mm thick sheets of grade 2 unalloyed Ti (ASTM F67 “Unalloyed Ti for surgical implant applications”) and provided by Institut Straumann AG (Basel, Switzerland). Disks punched to 15 mm in diameter to fit snugly into the well of a 24-well tissue culture plate. The fabrication method and characterization of the resulting morphology have been reported previously [20,21]. Briefly, smooth Ti surfaces (PT) have a mean peak-to-valley roughness (R_a) of 40 nm. To create SLA surfaces, PT surfaces are grit blasted and acid etched to create topography with craters (100 μ m diameter) overlaid with pits (1–3 μ m diameter) and coated with spikes (700 nm diameter). The resulting surface has a R_a of 3.2 μ m. SLA surfaces fabricated in a nitrogen environment to prevent exposure to air, and packaged in a sealed glass tube with isotonic saline to minimize exposure to the ambient atmosphere, thus retaining high surface energy, to create the modSLA surface. Disks were sterilized by gamma irradiation at 25 kGy overnight.

2.3 Quantification of Secreted BMPs

MSCs were plated on tissue culture polystyrene (TCPS) or on Ti disks (PT, SLA, or modSLA). Media were changed 24 hours after plating and every 48 hours thereafter until cultures reached confluence on TCPS, typically after 7 days of culture. At confluence, cells were incubated with fresh media for 24 hours. Secreted osteocalcin, BMP2, BMP4, and BMP7 levels were measured in the collected conditioned media as described below. Results are presented as normalized to total cell number.

2.4 BMP mRNA Levels

MSCs were plated on TCPS or on Ti disks. For studies at confluence, RNA was isolated from cultures (TRIzol® Reagent, Life Technologies, Carlsbad, CA). RNA was quantified by spectrophotometer (NanoDrop, Thermo Scientific, Waltham, MA) and 250 ng RNA reverse transcribed using random primers (High Capacity cDNA Reverse Transcription kit, Life Technologies). For time course studies, RNA was isolated from cells after 2, 4, or 6 days in culture (Ambion RNAqueous®-Micro Kit, Life Technologies) and 125 ng RNA reverse transcribed as above.

Levels of mRNA were quantified with real-time quantitative PCR (StepOnePlus, Life Technologies) using gene-specific primers. Primers (Table 1) were designed using Beacon designer software and synthesized by Eurofins MWG Operon (Huntsville, AL); the primers for Noggin were purchased from Qiagen (QuantiTect Primer Assay, QT00210833). Fluorescence values were related to starting mRNA quantities using known dilutions of MSCs grown on TCPS. mRNA levels for each gene are presented as normalized to mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.5 BMP2 Silencing

MSCs were transduced with shRNA lentiviral particles (NM_001200 TRCN0000058193, Mission®, Sigma Aldrich, St. Louis, MO) to establish stably silenced MSC cultures (shBMP2-MSC). MSCs were plated at 20,000 cells per cm^2 and cultured overnight in MSCGM. Particles were added to the cells at a multiplicity of infection of 5.0 in MSCGM

supplemented with 8 mg/ml hexadimethrine bromide (Sigma Aldrich) and incubated for 18 hours. After incubation, transduced cells were selected with MSCGM containing 0.25 µg/ml puromycin. Silencing of BMP2 was confirmed using real-time qPCR and ELISA as described. MSCs or shBMP2 MSCs were cultured on TCPS or Ti substrates. Culture medium was changed 24 hours after plating and every 48 hours thereafter until cultures reached confluence on TCPS. Cells were incubated with fresh MSCGM for 24 hours. Conditioned media and cell lysates were harvested and assayed as described below.

2.6 Effect of Exogenous BMP2

MSCs were cultured on TCPS or Ti. Cultures were fed daily with MSCGM supplemented with 40 ng/ml BMP2 (R&D Systems, Minneapolis, Minnesota). At confluence, cells were incubated with fresh MSCGM for 24 hours. Conditioned media and cell lysates were harvested and assayed as described below.

2.7 Antibody Blocking Studies

The effect of BMP inhibitors on MSC differentiation was assessed using culture media supplemented with blocking antibodies to specific secreted inhibitors. MSCs were cultured on TCPS or Ti. Cultures were fed daily with MSCGM containing 1 µg/ml of antibodies against Cerberus (Ab-Cerberus), Chordin (Ab-Chordin), Gremlin 1 (Ab-Gremlin), or Noggin (Ab-Noggin) (R&D Systems). At confluence, cells were incubated with fresh MSCGM for 24 hours. Conditioned media and cell lysates were harvested and were assayed as described below.

2.8 Biochemical Analysis

At harvest, cells were released from TCPS and Ti surfaces by two sequential ten-minute incubations in 0.25% trypsin-EDTA (Invitrogen) [20]. Total cell number was determined using a Z2 Particle Counter (Beckman Coulter, Brea, CA). Cells were lysed in 0.05% Triton X-100 (Sigma Aldrich, St. Louis, MO). Alkaline phosphatase specific activity was measured in the cell lysate and normalized to total protein content (Thermo Scientific Pierce BCA Protein Assay Kit, Rockford, IL) as described previously [20].

2.9 Quantification of Secreted Factors

Levels of secreted factors were measured in conditioned media by immunoassay. Osteocalcin was measured using a commercially available radioimmunoassay following manufacturer's instructions (Biomedical Technologies Inc., Stoughton, MA). BMP2 (PeproTech, Rocky Hill, NJ), BMP4 (R&D Systems), and BMP7 (R&D Systems) levels were measured by enzyme-linked immunosorbent assay (ELISA) following manufacturer's instructions. A custom indirect ELISA using a monoclonal anti-mouse Noggin primary antibody (MAB719, R&D Systems) and Peroxidase-AffiniPure Goat Anti-Rat IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) measured secreted Noggin. Known dilutions of recombinant human Noggin/Fc Chimera (R&D Systems) were used to generate a standard curve to extrapolate concentrations in experimental samples. Levels of osteoprotegerin, vascular endothelial growth factor

(VEGF), interleukin 6 (IL6), IL8, and IL10 were measured by commercially available ELISA (R&D Systems). Results of immunoassays were normalized to total cell number.

2.10 Statistical Analysis

Data are presented from one of two sets of experiments with comparable results (data from the second set of experiments are included in Supplemental Figures 1–7). Each variable is presented as mean + SEM of six independent cultures. Data were examined by analysis of variance (ANOVA) and significant differences between groups determined using Bonferroni's modification of Student's t-test. $P < 0.05$ was considered to be significant.

3. RESULTS

3.1 MSCs Produce BMP Molecules on Microtextured Surfaces

MSCs cultured on SLA exhibited higher osteocalcin production than cells on smooth PT (Fig. 1A), an effect further enhanced on modSLA substrates, confirming osteoblastic differentiation in the absence of exogenous factors as published previously [18]. Cells on SLA surfaces secreted 100% more BMP2 than on TCPS or PT, an effect enhanced on modSLA surfaces (Fig. 1B). Cells on SLA and modSLA surfaces secreted 100% more BMP4 (Fig. 1C) and BMP7 (Fig. 1D).

Surface roughness affected mRNA levels of BMP ligands. At confluence on TCPS, BMP2 (Fig. 2A) and BMP7 (Fig. 2C) mRNA levels were higher in MSCs cultured on rough or rough hydrophilic surfaces in comparison to TCPS or PT. Levels of BMP4 mRNA (Fig. 2B) were increased in response to surface microstructure. However, this differential expression was not evident at early time points during differentiation. BMP2 mRNA was similar on TCPS or Ti surfaces after 2 days of culture (Fig. 2D). After 4 days, levels were higher on rough SLA and modSLA than on PT or TCPS. By day 6, mRNA levels of BMP2 were greatly increased by surface roughness energy (TCPS < PT < SLA < modSLA). Temporal regulation of BMP4 mRNA levels occurred in a similar manner (Fig. 2E). BMP7 mRNA levels were similar on all surfaces on day 2 and only slightly increased on modSLA on day 4 in comparison to day 2 (Fig. 2F). However, levels were strongly increased on SLA and modSLA on day 6, particularly in comparison to smooth PT.

3.2 Osteogenic Differentiation of MSCs can be Altered by BMP Modulation

The effect of BMP2 on MSC differentiation was assessed in shBMP2-MSCs, in which BMP2 levels were decreased by more than 80% from wild type (WT) MSCs (Fig. 3A). shBMP2-MSCs had similar cell number to WT MSCs on TCPS and PT, but higher cell number on SLA and modSLA surfaces (Fig. 3B). Alkaline phosphatase specific activity was 20% lower in shBMP2-MSCs on SLA and modSLA than WT MSCs (Fig. 3C). Osteocalcin was reduced 15% in shBMP2-MSCs cultured on PT than WT MSCs, but was nearly 50% lower on SLA and modSLA surfaces (Fig. 3D). Silencing BMP2 in MSCs reduced osteoprotegerin levels on all substrates (Fig. 3E). VEGF secretion was lower in shBMP2-MSCs than WT MSCs cultured on Ti substrates, an effect enhanced on rough surfaces (Fig. 3F).

Exogenous BMP had only modest effects on MSC proliferation and differentiation. Addition of BMP2 to MSCs decreased cell number by 20% on all substrates (Fig. 4A). Alkaline phosphatase specific activity (Fig. 4B) and osteocalcin (Fig. 4C) were increased on all surfaces with BMP2 addition. Osteoprotegerin levels were decreased in a surface-roughness dependent manner in BMP2-treated cells (Fig. 4D). Addition of BMP2 did not affect levels of VEGF (Fig. 4E).

3.3 MSCs Produce BMP Antagonists on Microtextured Surfaces

MSCs also displayed differential regulation of BMP inhibitors during differentiation on microstructured Ti surfaces. At confluence on TCPS, mRNA for BMP inhibitors NOG (Fig. 5A), GREM1 (Fig. 5B), and CER1 (Fig. 5C) were higher on rough SLA than on TCPS or PT, and were further increased on modSLA surfaces. Culture on Ti surfaces had no effect on CHRD mRNA levels (Fig. 5D). Inhibitors were regulated temporally by surface topography. NOG was lower on SLA and modSLA after 2 days of culture than on TCPS or PT (Fig. 5E). However, mRNA levels were higher on SLA or modSLA than PT or TCPS after 4 days. NOG was 50% higher on SLA and modSLA on day 6 than on PT or TCPS. GREM1 mRNAs were the same on all surfaces at both day 2 and day 4 (Fig. 5F). However, GREM1 was 100% higher in MSCs on SLA or modSLA on day 6 than on TCPS or PT. Likewise, CER1 mRNA levels were similar on all surfaces at day 2 and day 4 (Fig. 5G). However, by day 6, levels were higher on rough SLA and modSLA surfaces than on smooth surfaces. CHRD mRNA levels were not different at the time points examined (Fig. 5H).

3.4 Osteogenic Differentiation of MSCs can be Altered by BMP Inhibitor Modulation

Modulation of specific BMP inhibitors enhanced differentiation of MSCs. Blocking antibodies against Cerberus, Gremlin1, Noggin, but not Chordin, decreased cell number on rough SLA and modSLA surfaces in comparison to untreated cells (Fig. 6A). Cells cultured with Ab-Cerberus had higher alkaline phosphatase specific activity on PT and SLA surfaces than untreated cells, while Ab-Gremlin and Ab-Noggin increased enzyme activity on all Ti surfaces (Fig. 6B). Ab-Cerberus increased osteocalcin secretion only on PT, while Ab-Gremlin and Ab-Noggin enhanced production on all Ti surfaces (Fig. 6C). Osteoprotegerin secretion was increased in Ab-Cerberus treated cells on PT and SLA, while Ab-Gremlin and Ab-Noggin treatment increased secretion on PT, SLA, and modSLA (Fig. 6D). However, Ab-Chordin treatment had no effect on these parameters (Fig. 6B–6D). Ab-Cerberus and Ab-Noggin increased VEGF production on rough SLA surfaces, while Ab-Chordin had no effect (Fig. 6E). Addition of Ab-Gremlin inhibited VEGF secretion on all surfaces, an effect enhanced on rough surfaces.

3.5 Interleukin Production by MSCs can be Altered by BMP Inhibitor Modulation

Modulation of BMP inhibitors also affected levels of secreted ILs, differing in profile than that elicited by addition of BMP2. Levels of IL6 secretion by MSCs decreased on rough surfaces in comparison to smooth (Fig. 7A). On smooth surfaces, Ab-Cerberus yielded lower levels of IL6 than control, but levels were 40% higher than control on rough surfaces. Ab-Gremlin decreased IL6 levels on smooth surfaces in comparison to control, but had no effect on rough SLA and modSLA. Ab-Noggin had no effect on IL6 secretion. Addition of BMP2 increased IL6 levels only on rough surfaces. IL8 decreased in MSCs on rough

surfaces from levels on smooth substrates (Fig. 7B). Ab-Cerberus increased IL8 levels on all substrates, with the greatest effect on rough surfaces. Ab-Gremlin slightly decreased IL8 levels only on smooth surfaces. Ab-Noggin modestly decreased IL8 levels on TCPS, PT, and SLA surfaces. BMP2 treatment increased IL8 on all surfaces, inducing 4-fold increases on rough surfaces. IL10 levels were higher on rough surfaces than smooth (Fig. 7C). Ab-Cerberus decreased IL10 levels slightly on all surfaces. Ab-Gremlin had no effect on IL10 secretion. Ab-Noggin increased IL10 levels on all surfaces. Addition of BMP2 decreased IL10, an effect that was greatest on rough surfaces.

4. DISCUSSION

Ti implants with microstructured, hydrophilic surfaces increase MSC differentiation [18] and peri-implant bone formation [16]. Our lab has previously demonstrated that in the absence of exogenous growth factors or stimulatory media, surface topography and surface energy are sufficient to induce MSC differentiation [18]. However, the precise molecular mechanisms that control this process are still unclear. Here we demonstrate tight regulation of BMP signaling during osteoblastic differentiation of MSCs on microstructured Ti surfaces, and modulation of these signals can enhance or inhibit this process.

We and others have demonstrated that surface roughness at the micron-submicron level enhances osteoblast maturation and induces MSC differentiation to an osteoblastic phenotype. We have studied the possible biological mechanisms, but how exactly surface roughness induces MSC differentiation is still unclear. It has been suggested that the increase in surface area can be translated into more cell-material contact; however, this hypothesis was rejected in a recent report demonstrating that cells grown on rough surfaces spread less and have less contact with the biomaterial surface than cells grown on smooth biomaterials [22].

In this study, we present evidence that BMP ligands (BMP2, BMP4, and BMP7) are upregulated temporally during MSC differentiation on microstructured Ti substrates. In our study, cells on rough Ti surfaces increased production of BMP2 and BMP4 mRNA as early as 4 days after plating, even in the absence of media supplements to induce osteoblast differentiation. These results indicate that BMP2 and BMP4 production is consequence of the surface topography and not due to factors present in osteogenic medium. In addition, our results suggest that BMP2 and BMP4 are early regulators of osteogenesis on these surfaces. BMP2 and BMP4 are crucial during embryonic bone formation and adult skeletal homeostasis [23,24], supporting the idea that BMP2 and BMP4 are necessary for early commitment to osteogenesis. Increase in secretion of these factors suggests that differentiation of MSCs on microstructured Ti surfaces occurs through autocrine and paracrine morphogen regulation [25], and provides a mechanism for the differentiation of MSCs distal to the implant surface seen in our previous studies [18]. These osteogenic factors are upregulated early after the initiation of culture on rough hydrophilic surfaces, providing molecular evidence of increased osteoblastogenesis seen *in vitro* [18] and for reduced time to loading and healing times seen clinically [16,26].

We previously reported that integrin subunits $\alpha 2$ and $\beta 1$ play an important role in osteoblastic differentiation, and inhibition of these integrins abolished the osteoblastic phenotype induced by biomaterials surface microstructures [27,28]. Additionally, we reported that members of the Wnt family such as Dkk1, Dkk2, and Wnt5a play important roles in controlling mesenchymal stem cell differentiation on microstructured biomaterials [29,30]. Interestingly, BMP2 gene expression increases at a time similar to the increase in WNT5A we previously reported in response to surface microarchitecture, and blocking integrin $\alpha 2$ expression results in an inhibition of WNT5A or BMP2 expression [30]. Taken together, our results suggest a complex crosstalk between integrins, Wnt5a, and BMP2 that must be elucidated further. An advantageous feature of substrate-induced BMP2 activation is its synchronization with other adhesion-associated events, eliciting more coordinated substrate-driven interactions with molecules that impinge upon the BMP2 pathway.

Endogenous BMP2 is involved in bone repair and regeneration. Limb-specific deletion of BMP2 in mice prevents fracture healing [31] and activation of endogenous BMP2 appears to play a role in distraction osteogenesis [32]. To understand the role that BMP2 plays in the osteogenic effects of Ti surface microtopography, we reduced BMP signaling using BMP2-silenced MSCs, which reduced their osteoblast differentiation and local factor production on microstructured Ti. However, silencing BMP2 yielded only partial attenuation of downstream surface effects, indicating that other signaling may compensate in its absence. We selected reduction of BMP2 over other BMPs since BMP2 addition is the most commonly used clinically. Addition of BMP2 increased markers of osteoblast differentiation as expected, but decreased osteoprotegerin levels, an important regulator of osteoclastogenesis [33]. These results suggest that BMP2 may also regulate bone remodeling by controlling osteoprotegerin production; however, effects on osteoclast biology were not explored here.

Cells regulate BMP signaling through receptor availability, pseudoreceptors, and cytoplasmic and secreted inhibitors. Extracellular BMP antagonists regulate osteoblast differentiation and osteogenesis [34]. Several studies have shown that addition or overexpression of Noggin, Gremlin1, Chordin, or Twisted impairs osteoblast differentiation *in vitro* and *in vivo* bone formation [4,35]. Conversely, inhibition of Noggin, Sclerostin, or Chordin enhances osteoblast differentiation *in vitro* and bone formation *in vivo* [17,19,36]. In our study, levels of Noggin decreased on rough surfaces on day 2 of culture, suggesting this reduction may be responsible for initial osteogenic induction. Interestingly, mRNAs for Noggin increased after 4 days of culture, at the same that BMP2 and BMP4. BMP2, BMP4, and BMP6 upregulate Noggin expression, suggesting there is negative feedback controlling BMP pathway activation [37].

Multiple BMP antagonists are present in bone formation and healing; thus, it is plausible that inhibition of one or multiple BMP antagonists increase(s) available endogenous BMP proteins that can enhance osteoblast differentiation of MSCs on microstructured Ti surfaces. Our results confirm this hypothesis, since reducing endogenous Noggin with blocking antibodies enhanced osteoblast markers on microstructured Ti surfaces. Our findings confirm previous studies using RNA interference to reduce BMP inhibitors Noggin [17] and Chordin [19], which showed increased osteoblastic differentiation of MSCs and increased

bone formation in vivo. Cerberus and Gremlin1 also play a role in bone formation and fracture healing [38], and reducing their effects via blocking antibodies enhanced osteoblast differentiation on the microtextured Ti surfaces in our study.

A potential advantage of substrate-stimulated activation of endogenous BMP2 versus treatment with exogenous BMP2 concerns the latter's drawback of co-stimulating inflammatory processes. Exogenous BMP2 has been demonstrated to induce bone formation, but adverse effects including ectopic bone formation, osteolysis, and seroma formation have been reported [11,12]. Soft tissue inflammation was seen as early as 3 hours after subcutaneous BMP2 implantation in a rat model, an effect dependent on BMP2 dose [39,40]. We previously reported that exogenous BMP2 induced pro-inflammatory interleukin production by osteoblast-like cells on microstructured Ti surfaces in a TAB/TAK-dependent manner [13]. In the present study, MSCs on microtextured Ti produced lower levels of pro-inflammatory IL6 and IL8 and higher levels of anti-inflammatory IL10 than MSCs on smooth Ti or TCPS. However, addition of exogenous BMP2 reversed or blocked the effect of surface microtopography, increasing pro-inflammatory IL6 and IL8 and decreasing anti-inflammatory IL10.

We also examined the role secreted BMP antagonists had on induction of inflammatory interleukins. Blocking antibodies against secreted BMP inhibitors yielded differentiation state-dependent changes in IL production. Ab-Cerberus increased pro-inflammatory interleukin production on rough surfaces; IL-6 and IL-8 expression increased to levels equivalent to those induced by exogenous BMP2 treatment. In contrast, Ab-Noggin increased anti-inflammatory IL10 production in all conditions. Thus when adopting a strategy of blocking negative-feedback regulators to enhance substrate-activated endogenous BMP2 signaling, careful attention needs to be made regarding the best complementary agent to mitigate the potential deleterious side-effect of stimulating inflammatory cytokines.

BMP inhibitors bind BMP ligands with differing affinity, which may explain their diverse roles in modulating MSC osteogenesis and BMP-stimulated interleukin production. BMP ligands can form heterodimers or homodimers, activating downstream signaling [41]. It is likely that in vivo, BMPs function mainly as heterodimers to induce controlled bone formation. In the presence of exogenous BMP2, or when specific inhibitors are blocked resulting in excess BMP levels, we hypothesize that BMP molecules form homodimers that initiate signaling cascades that end in inflammation, bone resorption, and apoptosis. Taken together, the data suggest that modulating endogenous BMP inhibitors may be an effective approach to control bone formation while limiting the pro-inflammatory environment induced by BMP2 application. The present study did not determine if blocking inhibitors with antibodies might ultimately result in higher endogenous BMP2 expression, as was the case when Noggin was knocked-down in MG63 osteoblast-like cells cultured on microstructured Ti surfaces [15]. The phenotypic outcomes from BMP inhibitor-blocking antibodies were alkaline phosphatase activity and secreted osteocalcin, osteoprotegerin, and VEGF. Although downstream of BMP2 activity, these biomarkers can be influenced by other pathways such as FGF-2 or Wnt signaling. However, using a reporter plasmid to measure the activity of bone morphogenetic protein signaling [42] could provide a more

sensitive and direct approach to confirm that blocking extracellular BMP inhibitor proteins with antibodies serves to enhance BMP signaling per se.

Our results support the conclusion that blocking secreted BMP inhibitors can exploit the endogenous BMP2 production by MSCs in response to microstructured Ti. However, others have found that Noggin suppression resulted in poorer BMP2-induced osteogenesis [43]. The ultimate outcome may also be influenced by surface nanotopography [44], the response of other cell types around the implant to BMP2, and prove to be context-dependent. Moreover, a number of mechanisms may be responsible for an apparent BMP2 enhancement when MSCs are cultured on microtextured Ti, including the expression of microRNAs that influence BMP2 pathways [45–47]. The extent to which blocking extracellular BMP2 inhibitors can enhance osteogenesis in the context of microtextured Ti remains to be tested *in vivo*.

5. CONCLUSIONS

The results demonstrate that the BMP signaling is critical for MSC osteoblastogenesis, displaying tight regulation of BMP ligands and inhibitors on microstructured Ti. This pathway can be stimulated with exogenous BMP2 to enhance osteoblastic differentiation, but this approach increased important pro-inflammatory cytokines. Secreted BMP inhibitors can be blocked to exploit endogenous production of BMP2, BMP4, and BMP7 to increase osteoblastic differentiation. This study demonstrates the importance of BMP signaling in MSC osteoblastic differentiation on microstructured Ti surfaces and alteration of these molecules can negatively affect osseointegration and inflammatory cytokine production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Tight regulation of BMP ligands and inhibitors occurs during MSC osteogenesis on microstructured Ti.
- Osteoblastogenesis and pro-inflammatory cytokine production increased with exogenous BMP2.
- Blocking secreted BMP inhibitors exploits endogenous BMP production, increasing osteoblastic differentiation without increasing inflammatory cytokines.

Figure 1. Secretion of BMP ligands from MSCs cultured on microstructured titanium surfaces MSCs were cultured on TCPS, PT, SLA, and modSLA surfaces. Secreted osteocalcin (A), BMP2 (B), BMP4 (C), and BMP7 (D) were measured in the conditioned media. * $p < 0.05$, Ti surface versus TCPS; # $p < 0.05$, versus PT; \$ $p < 0.05$, versus SLA.

Figure 2. Levels of mRNA for BMP pathway ligands in MSCs cultured on microstructured titanium surfaces

Levels of mRNA for BMP2 (A), BMP4 (B), and BMP7 (C) were measured in MSCs at confluence on TCPS. * $p < 0.05$, Ti surface versus TCPS; # $p < 0.05$, versus PT; \$ $p < 0.05$, versus SLA. Levels of mRNA for BMP2 (D), BMP4 (E), or BMP7 (F) were measured after 2, 4, or 6 days of culture on TCPS or Ti substrates. * $p < 0.05$, Ti surface versus TCPS; # $p < 0.05$, versus day 2; \$ $p < 0.05$, versus day 4.

Figure 3. Effect of BMP2 silencing on MSC differentiation on microstructured titanium surfaces Wild type (WT) MSCs or shBMP2-MSCs were plated on TCPS, PT, SLA, and modSLA surfaces and cultured to confluence on TCPS. Levels of BMP2 (A), cell number (B), alkaline phosphatase specific activity (C), osteocalcin (D), osteoprotegerin (E), and VEGF (F) were measured 24 hours after confluence. * $p < 0.05$, Ti surface versus TCPS; # $p < 0.05$, shBMP2-MSCs versus wild type (WT).

Figure 4. Effect of exogenous BMP2 on MSC differentiation on microstructured titanium surfaces

MSCs were plated on TCPS, PT, SLA, and modSLA surfaces and cultured to confluence on TCPS in MSCGM or MSCGM supplemented with 40 ng/ml rhBMP2. Cell number (A), alkaline phosphatase specific activity (B), osteocalcin (C), osteoprotegerin (D), and VEGF (E) were measured 24 hours cells reached confluence on TCPS. * $p < 0.05$, Ti surface versus TCPS; # $p < 0.05$, versus untreated cells.

Figure 5. Levels of mRNA for BMP pathway inhibitors in MSCs cultured on microstructured titanium surfaces

Levels of mRNA for Noggin (NOG, A), Gremlin1 (GREM1, B), Cerberus (CER1, C), and Chordin (CHR1, D) were measured in MSCs at confluence on TCPS. * $p < 0.05$, Ti surface versus TCPS; # $p < 0.05$, versus PT; \$ $p < 0.05$, versus SLA. Levels of mRNA for NOG (E), GREM1 (F), CER1 (G), and CHR1 (H) were measured after 2, 4, or 6 days of culture on TCPS or Ti substrates. * $p < 0.05$, Ti surface versus TCPS; # $p < 0.05$, versus day 2; \$ $p < 0.05$, versus day 4.

Figure 6. Effect of BMP inhibitor modulation on MSC differentiation on microstructured titanium surfaces

MSCs were plated on TCPS, PT, SLA, and modSLA surfaces and treated daily with antibodies raised against Cerberus, Chordin, Gremlin1, or Noggin. Cell number (A), alkaline phosphatase specific activity (B), osteocalcin (C), osteoprotegerin (D), and VEGF (E) were measured 24 hours after cells reached confluence on TCPS. * $p < 0.05$, Ti surface versus TCPS; # $p < 0.05$, versus untreated cells.

Figure 7. Effect of BMP inhibitor modulation on MSC interleukin production on microstructured titanium surfaces

MSCs were plated on TCPS, PT, SLA, and modSLA surfaces and treated daily with Ab-Cerberus, Ab-Gremlin1, Ab-Noggin, or with BMP2 protein. Levels of IL6 (A), IL8 (B), and IL10 (C) were measured 24 hours after cells reached confluence on TCPS. * $p < 0.05$, Ti surface versus TCPS; # $p < 0.05$, versus untreated cells.

Table 1

Primer sequences used in real-time qPCR analysis

Gene	NCBI Reference Sequence	Amplicon Size	Primer Sequence	
BMP2	NM_001200.2	126	F	GCG TGA AAA GAG AGA CTG C
			R	CCA TTG AAA GAG CGT CCA C
BMP4	NM_001202.3	105	F	ACG GTG GGA AAC TTT TGA TGT G
			R	CGA GTC TGA TGG AGG TGA GTC
BMP7	NM_001719.2	114	F	AGC AGC AGC GAC CAG AGG
			R	ACA GTA GTA GGC GGC GTA GC
CER1	NM_005454.2	133	F	TAC CTC CTG CTC TCA CTG TT G
			R	ATG CTC CGT CTT CAC CTT GC
CHRD	NM_003741.2	184	F	AGC CCC AGA GAC CAG AAC T
			R	GGG AGT AGA GGC AGG ACA GA
GAPDH	NM_002046.5	188	F	GCT CTC CAG AAC ATC ATC C
			R	TGC TTC ACC ACC TTC TTG
GREM1	NM_013372.6	117	F	GCA GGG TGG GTG AAC TTT ATT G
			R	AGG AGG CTG AGA AGA TAC AAG G