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Microstructured Titanium Regulates Interleukin Production by Osteoblasts, an Effect Modulated by Exogenous BMP-2

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Abstract

Microtextured implant surfaces increase osteoblast differentiation in vitro and enhance bone-to-implant contact in vivo and clinically. These implants may be used in combination with recombinant human bone morphogenetic protein 2 (rhBMP-2) to enhance peri-implant bone formation. However, the effect of surface modifications alone or in combination with rhBMP-2 on osteoblast-produced inflammatory microenvironment is unknown. MG63 cells were cultured on tissue culture polystyrene or titanium substrates: smooth pretreated (PT, Ra=0.2μm), sandblasted/acid-etched (SLA, Ra=3.2μm), or hydrophilic-SLA (modSLA). Expression and protein production of pro-inflammatory interleukins (IL1b, IL6, IL8, IL17) and anti-inflammatory interleukins (IL10) were measured in cells with or without rhBMP-2. To determine which BMP signaling pathways were involved, cultures were incubated with BMP pathway inhibitors to blocking Smad (dorsomorphin), TAB/TAK1 ((5Z)-7-oxozeaenol), or PKA (H-8) signaling. Culture on rough SLA and modSLA surfaces decreased pro-inflammatory interleukins and increased anti-inflammatory IL10. This effect was negated in cells treated with rhBMP-2, which caused an increase in pro-inflammatory interleukins and a decrease in anti-inflammatory interleukins through TAB/TAK signaling. The results suggest that surface microtexture modulates the inflammatory process during osseointegration, an effect that may enhance healing. However, rhBMP-2 in combination with microtextured titanium implants can influence the effect of cells on these surfaces, and may adversely affect cells involved in osseointegration.

Keywords

Microstructure; Inflammation; BMP (bone morphogenetic protein); Titanium

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DISCLOSURES

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1. INTRODUCTION

Biocompatibility of materials is an important consideration in the development of dental and orthopaedic implants, as this factor can greatly affect osseointegration and implant success. Cells interact with the surface of the implant and these interactions dictate the response of the cells to the biomaterial and lead to osseointegration of the implant to the bone. Surface properties have been modified to increase osteoblast differentiation and bone formation. It has been shown that microtextured titanium surfaces promote osteoblast differentiation and that high surface energy, achieved by controlling surface hydrocarbon contamination, further increases osteoblast differentiation, maturation, and bone formation around implants [1, 2].

Peri-implant osteogenesis requires net new bone formation. While stimulatory effects on osteoblast maturation are important, control of osteoclastic remodeling is also critical. Several studies have shown that osteoblasts contribute to this process by producing higher levels of osteoprotegerin (OPG) [3, 4], a decoy receptor for RANK ligand, thereby potentially reducing recruitment and activation of osteoclasts [3]. Inflammation processes play a role as well, particularly during the acute inflammatory response triggered in the early periods after injury [5, 6]. These initial inflammatory events initiate mesenchymal stem cell recruitment and subsequent tissue repair [7-9]. Regulation of bone formation and subsequent bone remodeling can also be adversely affected by inflammatory responses, however, causing a shift from bone formation towards osteoclastogenesis and bone resorption that could impair osseointegration or lead to implant failure [10-13].

In vivo studies indicate that inflammation plays a role in osseointegration of metallic implants [14-16]. We recently reported that dendritic cell maturation and factor production is regulated on microtextured Ti implant surfaces with high wettability [17], indicating that immune cells are sensitive to biomaterial surface properties. However, production of inflammatory cytokines is not limited to immune cells. Osteoblasts produce abundant cytokines to regulate inflammation, which include interleukins (IL). ILs can be classified as pro- or anti-inflammatory, although many can exhibit both types of behavior depending on the concentration. Pro-inflammatory interleukins may promote inflammation [18] or activate osteoclasts [19], and some have been demonstrated to play important roles in inflammatory bone diseases [20]. One of the most powerful anti-inflammatory cytokines, IL10 [21], inhibits TH₁ cytokines, monocytes, macrophages, and neutrophils [22], modulating acute inflammation and promoting humoral T_H2 immune responses. Previous studies have demonstrated that osteoblast-like MG63 cells produce pro-inflammatory cytokines after various stimuli, including orthopaedic materials [23-25].

Interleukins work together with many other cytokines in a complex network that determines the overall inflammatory response of the host to an implanted biomaterial. However, determining the host-material interaction becomes more complicated as exogenous factors are introduced to enhance bone apposition. Bone morphogenetic proteins (BMPs) induce bone formation in development [26]. Recombinant human BMP-2 (rhBMP-2) is used clinically to induce bone formation in hard-to-heal fractures and defects, and is now suggested to be used in combination with metallic biomaterials to induce peri-implant bone regeneration [27-30]. Although this growth factor has been demonstrated to increase osteogenesis in the lumbar spine [31, 32], inflammatory complications including seroma and osteolysis have been reported [33-36]. In vivo addition of rhBMP-2 also induces inflammation [37, 38]. There are a number of possible reasons for this, but the local concentration of BMP-2 may be a contributing factor [34].

While cytokine production is necessary for inflammatory responses to regulate the healing process, an imbalanced pro-inflammatory response after biomaterial implantation can produce extreme inflammation and possibly compromise implant success. Little is known about the effects of the surface properties of an implant and BMP-2 on the inflammatory microenvironment created by osteoblasts during osseointegration. The aim of the current study was to determine whether pro-osteogenic surface properties modulate the inflammatory interleukin response of osteoblasts in vitro and determine the effect exogenous rhBMP-2 has on this process.

2. MATERIALS AND METHODS

2.1 Ti Disk Preparation and Cell Culture

Ti disks 15mm in diameter were prepared from 1mm thick sheets of grade 2 commercially pure Ti and supplied to us by Institut Straumann AG (Basel, Switzerland). The methods used to produce smooth pretreatment (PT), rough sandblasted acid etched (SLA), and hydrophilic SLA (modSLA) have been reported previously [39]. Surface roughness was determined by laser confocal microscopy (LEXT 3D Material Microscope, Olympus America, Center Valley, PA) using a 100X objective and $128\ \mu\text{m} \times 128\ \mu\text{m}$ field of view [PT, $R_a = 0.21 \pm 0.01\ \mu\text{m}$; SLA $R_a = 3.22 \pm 0.34\ \mu\text{m}$, modSLA $R_a = 3.32 \pm 0.62\ \mu\text{m}$]. Contact angle measurements were determined using a Ramé-Hart goniometer and DROPimage Software (Ramé-Hart Instrument Company, Netcong, NJ) [PT- $92^\circ \pm 2^\circ$; SLA $128^\circ \pm 3^\circ$; modSLA- 0°].

Human MG63 osteoblast-like osteosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD). These cells are a progenitor cell model of osteoblast differentiation used in biomaterial studies, and have been previously demonstrated to increase markers of osteoblast maturation in response to Ti substrates with micron- and submicron-scale roughness and high surface energy [40, 41]. In addition, they are used as a cell culture model to test cytokine production stimulated by orthopaedic materials [23-25]. MG63 cells were plated on tissue culture polystyrene (TCPS) or Ti substrates at a density of 10,000 cells per cm^2 . Cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were cultured at 37°C and 100% humidity. Experiments were performed when cells reached confluence on TCPS, typically after four days of culture.

2.2 Interleukin Expression

IL mRNA was measured in cells cultured as described on TCPS or Ti substrates. At confluence on TCPS, cells on all surfaces were incubated with fresh medium for 12h and RNA harvested using a TRIzol® (Invitrogen, Carlsbad, CA) extraction method following manufacturer's protocol. mRNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). RNA (500 ng) was amplified using reverse transcription (High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Carlsbad, CA). Starting quantities of mRNA were determined using SybrGreen chemistry (Power SYBR® Green PCR Master Mix, Applied Biosystems) in a StepOne Plus imaging system (Applied Biosystems). MG63 cells grown on TCPS were used to generate a standard curve for each gene of interest and values for each sample extrapolated. mRNA was measured for IL1A, IL1B, IL6, IL7, IL8, IL10, and IL17 (sequences in Table 1) using gene specific primers (MWG Operon, Huntsville, AL). Osteocalcin mRNA (OCN) was measured as an indicator of osteoblast differentiation. All mRNAs are presented as normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.3 Interleukin Protein Production

Cells were cultured as described on TCPS or Ti disks and at confluence on TCPS, cells on all surfaces were incubated with fresh medium for 24h. Cells were released from TCPS and Ti surfaces using two sequential incubations with 0.25% trypsin for 10 min at 37°C and counted using a cell counter (Z2 Particle counter, Beckman Coulter, Fullerton, CA). Cellular alkaline phosphatase specific activity was measured to verify cell response to the microstructured Ti surfaces. Briefly, alkaline phosphatase specific activity (orthophosphoric monoester phosphohydrolase, alkaline; E.C. 3.1.3.1) was determined in cell lysates using a colorimetric assay measuring the conversion of *p*-nitrophenol from *p*-nitrophenylphosphate by alkaline phosphatase at 37°C [42]. Levels of secreted cytokines IL1a, IL6, IL8, IL10, and IL17 were assayed in the conditioned medium using ELISA (R&D Systems DuoSet, Minneapolis, MN). Protein levels were normalized to total cell number.

To determine the effect of rhBMP-2 on interleukin production, cells were cultured as described on TCPS or Ti substrates. At confluence on TCPS, cells were incubated with fresh medium $\pm$ 40ng/ml rhBMP-2 for 24 hours. Levels of pro-inflammatory (IL1a, IL6, IL8, IL17) and anti-inflammatory (IL10) interleukins were measured in the conditioned medium by ELISA as described above.

2.4 BMP Pathway Mediating Interleukin Expression

BMP-2 binds to tetramers of BMP receptors, initiating downstream signaling through canonical Smad signaling or non-canonical protein kinase A (PKA) or TAB/TAK1 signaling. To determine the BMP pathway involved in inflammatory cytokine expression, specific BMP pathway inhibitors were used. (5Z)-7-Oxozeaenol, a TAK1 inhibitor [43, 44], dorsomorphin, an inhibitor of type I BMP receptors (ALK2, ALK3, ALK6) that prevents Smad phosphorylation [45, 46], and H-8, a PKA inhibitor [47, 48], were purchased from EMD Chemicals (Gibbstown, NJ). Cells were cultured on modSLA substrates as described above. Cells were pre-incubated for 2 hours with control medium, 200 nM (5Z)-7-Oxozeaenol, 10 nM Dorsomorphin, or 5 μ M H-8 followed by treatment with inhibitor $\pm$ 40 ng/ml rhBMP-2 for 12 hours. RNA was harvested and mRNA levels quantified as described above.

2.5 Statistical Analysis

Data presented are from one of two sets of experiments, with comparable results. Each data point is the mean $\pm$ standard error of the mean (SEM) of six independent cultures. Data were analyzed by analysis of variance 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 Effect of Surface Roughness and Energy on Interleukin Expression

mRNAs for osteocalcin increased in a surface roughness and energy dependent manner (Fig. 1A), confirming increased cell maturation. mRNAs for anti-inflammatory IL10 increased with increasing surface roughness and energy, with a 3-fold increase on modSLA in comparison to TCPS or PT (Fig. 1B).

Expression of mRNAs for pro-inflammatory interleukins was also regulated by surface roughness and energy. IL1A expression was increased on rough surfaces (Fig. 2A). However, while IL1B mRNA was 1.5-times higher on the rough SLA surface than on TCPS or PT, levels on modSLA were similar to PT (Fig. 2B). IL6 mRNA decreased on SLA in comparison to TCPS or PT, but was lowest on modSLA (Fig. 2C). IL7 mRNA decreased on all titanium surfaces in comparison to TCPS (Fig. 2D). Levels were similar in cells grown

on PT or modSLA surfaces, but were highest on SLA. IL8 mRNA decreased on SLA and modSLA surfaces in comparison to TCPS or PT, with the lowest levels on modSLA substrates (Fig. 2E). However, mRNAs for IL17 decreased on rough SLA surfaces and was further lowered by culture on modSLA (Fig. 2F)

3.2 Effect of Surface Roughness and Energy on Interleukin Production

Cell number was lower on titanium surfaces than on TCPS, with further decreases on rough (SLA) and rough/hydrophilic surfaces (Fig. 3A). Alkaline phosphatase specific activity (Fig. 3B) increased in a roughness and surface energy-dependent manner (TCPS < PT < SLA < modSLA). These results confirm cell response to these materials as demonstrated previously [39].

Cells on Ti had higher IL10 production than on TCPS, but modSLA induced highest production of the anti-inflammatory IL10 (Fig. 4A). Surface roughness increased secreted IL1a by 100% in comparison to TCPS or PT surfaces, but levels on modSLA surfaces were 50% higher than on SLA (Fig. 4B). Levels of secreted IL6 and decreased in a roughness and surface energy-dependent manner, with the lowest levels produced on modSLA surfaces (Fig. 4C). IL8 production was similar on TCPS and PT surfaces, but levels were lower on SLA, and lowest on modSLA (Fig. 4D). Secreted IL17 was lower on PT and SLA surfaces than on TCPS; however, levels were lowest on modSLA (Fig. 4E).

3.3 Effect of Bone Morphogenetic Protein-2 on Interleukin Production

rhBMP-2 treatment had no effect on cell number on the surfaces examined (Fig. 5A). Alkaline phosphatase specific activity was higher in rhBMP-2 treated cells on SLA surfaces than in untreated cells (Fig. 5B). The increase in anti-inflammatory IL10 seen in cells cultured on hydrophilic rough surfaces was abolished by rhBMP-2 treatment (Fig. 6A). However, the increase in IL1a production by cells on rough surfaces was enhanced by BMP-2 treatment (Fig. 6B). While culture on rough or high-energy rough surfaces decreased production of IL6 and IL8, treatment with BMP-2 reversed this effect (Fig. 6C, 6D). Addition of BMP-2 to cells rough Ti caused a 100% increase in IL17 production in comparison to untreated cells (Fig. 6E).

3.4 Mechanism of BMP-2 Effect

Treatment with rhBMP-2 increased expression of MSX2, a transcription factor that is transcribed after induction of BMP-2 signaling (Fig. 7A). Treatment with dorsomorphin, but not (5Z)-7-oxozeaenol or H8, abolished this effect. All three inhibitors abolished the effect of rhBMP-2 treatment on mRNAs for IL10 (Fig. 7B). Increased levels of IL1a, IL6, and IL17 mRNAs after rhBMP-2 treatment was blocked with (5Z)-7-Oxozeaenol and Dorsomorphin, but not H8 (Fig. 7B, 7C, 7F). rhBMP-2 induced upregulation of IL8 expression was inhibited by all three inhibitors (Fig. 7E).

4. DISCUSSION

An inflammatory response is necessary for osseointegration [16]. However, inflammation must occur in a coordinated series of events to prevent skewing the response from bone formation to bone resorption, preventing osseointegration and leading to implant failure. In the current study, we examined the effect of Ti surface properties to modulate inflammatory interleukin production by osteoblasts. The results show that both surface topography and energy affect interleukin gene expression and protein levels in MG63 cells. Moreover, these effects are altered by rhBMP-2 through TAB/TAK signaling.

IL1a and IL1b expression and protein production was higher on rough and hydrophilic rough Ti surfaces than on smooth Ti. While IL1a and IL1b are pro-inflammatory cytokines, they also mediate bone formation. Mesenchymal stem cells treated with IL1b exhibit an osteoblastic phenotype, including formation of Alizarin red positive nodules [49]. Moreover, delivery of IL1b during the first 72 hours after fracture sped healing in an *in vivo* model [50]. In addition, IL1b expression is increased in bone during fracture healing [51]. Thus, an increase in these cytokines at low concentrations is consistent with enhanced bone healing.

Reduced expression and secretion of pro-inflammatory interleukins IL6, IL9, and IL17 was observed on rough Ti surfaces in comparison to smooth Ti. IL6 induces bone resorption by increasing osteoclast activity [52, 53] and levels of this interleukin are increased in aseptic loosening [54, 55]. IL8 is a powerful recruiter of neutrophils and is present in high levels in areas of polymorphonuclear infiltration [56] and in prostate cancer-induced osteolytic lesions [57]. There is evidence that IL17 modulates the OPG-RANKL balance, increasing osteoclastogenesis and inducing bone remodeling [58, 59]. *In vivo*, controlled secretion of osteoclast-activating interleukins by cells differentiating on the implant surface may help increase peri-implant bone formation by modulating osteoclast activity.

IL10 suppresses production of pro-inflammatory cytokines by immune cells [60, 61]. Interestingly, anti-inflammatory IL10 was increased on rougher surfaces, an effect more robust with increased surface energy. This suggests that rougher surfaces, particularly modSLA, promote an anti-inflammatory response. The results demonstrate that cells on microstructured Ti surfaces secrete factors to modulate bone formation, bone resorption, and inflammation, and this effect is enhanced on rough, high-energy surfaces.

Immune response to biomaterials, particularly in bone healing, is a complex process regulated by a number of cells of diverse lineages. In addition to direct effects on bone formation, it also has been shown that surface properties can affect other parameters that may affect implant success, including response of immune system cells to the biomaterial. Hydrophilic rough surfaces were found to support an immature dendritic phenotype while smooth surfaces enhanced dendritic cell maturation [17]. Macrophages cultured on the same hydrophilic rough surfaces expressed lower levels of pro-inflammatory cytokines than cells on smooth substrates [62].

Neutrophils are critical in the initial healing response, secreting interleukins and other chemokines that trigger the first wave of immune response. This initial inflammation typically resolves, but in the presence of stimulatory factors can continue, during which time neutrophils secrete matrix proteinases that can damage the host bone and compromise healing. Osteoprogenitor cells, in response to surface cues, can secrete anti-inflammatory factors that may help to prevent this degradation and speed transition of the immune response from an acute, neutrophil-mediated phase, allowing angiogenesis and bone formation to occur. We suggest that surface properties directly control the inflammatory microenvironment created by osteoblasts and activation of immune cells, and that this immuno-modulation may contribute to better and faster osseointegration seen clinically in modSLA implants.

In the present study, the effect of exogenous rhBMP-2 on inflammatory interleukin production was examined. Administration of rhBMP-2 to osteoblasts cultured on microstructured Ti implants increased pro-inflammatory, pro-osteoclastogenic interleukins and decreased the anti-inflammatory IL10. It is important to note that the effect of rhBMP-2 was not observed on TCPS, a material commonly used for *in vitro* studies, but that rhBMP-2 had a strong effect on cells grown on rough or hydrophilic rough surfaces. Interestingly,

treatment with BMP-2 abolished the positive effect of surface features on interleukins, inducing the opposite cytokine profile in comparison to surface roughness and energy alone.

Recombinant human BMP-2 is one of two clinically available BMPs approved for use to address the clinical need for bone formation during regenerative procedures. While this morphogen has been demonstrated to induce bone formation, there have been reports of adverse effects clinically including ectopic bone formation, osteolysis, and seroma formation [35, 36]. In animal models of inflammation, soft tissue inflammation was seen as early as 3 hours after subcutaneous implantation of rhBMP-2, an effect dependent on protein dose [37, 38]. The same study found increases in levels of secreted IL6. Studies have demonstrated that rhBMP-2 upregulates IL6 in normal human osteoblasts, confirming the present results [63]. Interestingly, in the dose used in our study, the increase in pro-inflammatory interleukin secretion came with only small gains in alkaline phosphatase specific activity, a marker of osteoblast maturation. We have demonstrated that 100 and 200 ng/ml rhBMP-2 induces apoptosis in MG63 and normal human osteoblasts, further confirming the dose-specific effects of rhBMP-2 [64].

While it has been demonstrated that rhBMP-2 can induce bone formation, it also has the potential to result in serious complications like bone resorption and swelling. Here, we examined the signaling pathways by which the rhBMP-2 increased interleukin production occurs. In our study, the TAB/TAK1 inhibitor (5Z)-7-Oxozeaenol blocked rhBMP-2 induced IL6 expression. TAK1 has been demonstrated to mediate IL-6 expression during cyclic stress in osteoblasts [65]. In addition, TAK1 silencing decreases the pro-inflammatory environment in a mouse inflammatory model [66]. The inflammatory environment induced by high doses of BMP-2 is likely due to the ability of BMP-2 to activate this signaling pathway. Interestingly, studies have found that rhBMP-2 directly induces osteoclast differentiation [67, 68]. Future clinical approaches to bone regeneration using rhBMP-2 would optimally activate only bone formation pathways and only minimally activate these signaling pathways that result in inflammation and bone resorption.

5. CONCLUSIONS

Taken together, the results suggest surface roughness and energy modulate the initial inflammatory response, increasing anti-inflammatory interleukins and reducing pro-inflammatory interleukins. Surfaces known to induce osteoblast differentiation also modulate interleukin production. The control of interleukins production and other inflammatory processes by surface features may have a major role in bone healing and osseointegration. However, BMP-2 treatment during titanium implant insertion in orthopaedic and dental applications may produce adverse effects through TAB/TAK signaling by reversing the surface effect on the inflammation process, increasing pro-inflammatory interleukins, which may delay the bone formation process.

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Figure 1. Effect of surface microstructure and energy on anti-inflammatory gene expression. MG63 cells were cultured on TCPS or Ti surfaces and expression of OCN (A) and IL10 (B) measured by real-time qPCR. * $p < 0.05$, vs. TCPS; # $p < 0.05$, vs. PT; \$ $p < 0.05$, vs. SLA.

Figure 2. Effect of surface microstructure and energy on pro-inflammatory interleukin expression of MG63 cells. MG63 cells were cultured on TCPS or Ti surfaces and expression of IL1A (A), IL1B (B), IL6 (C), IL7 (D), IL8 (E), and IL17 (F) measured by real-time qPCR. * $p < 0.05$, vs. TCPS; # $p < 0.05$, vs. PT; \$ $p < 0.05$, vs. SLA.

Figure 3. Effect of surface microstructure and energy on osteogenic differentiation of MG63 cells. MG63 cells were cultured on TCPS or Ti surfaces and cell number (A) and alkaline phosphatase specific activity (B) measured at confluence. * $p < 0.05$, vs. TCPS; # $p < 0.05$, vs. PT; \$ $p < 0.05$, vs. SLA.

Figure 4.

Effect of surface microstructure and energy on interleukin production by MG63 cells. MG63 cells were cultured on TCPS or Ti surfaces and production of anti-inflammatory IL10 (A) or pro-inflammatory IL1b (B), IL6 (C), IL8 (D), and IL17 (E) measured in the conditioned media. * $p < 0.05$, vs. TCPS; # $p < 0.05$, vs. PT; \$ $p < 0.05$, vs. SLA.

Figure 5.

Effect of rhBMP-2 on MG63 differentiation on microstructured Ti surfaces. MG63 cells were cultured on TCPS or Ti surfaces. At confluence, cells were treated with medium (control) or 40 ng/ml rhBMP-2 for 24 hours and cell number (A) or alkaline phosphatase specific activity (B) measured. * $p < 0.05$, vs. TCPS; # $p < 0.05$, vs. PT; \$ $p < 0.05$, vs. SLA; % $p < 0.05$, vs. control.

Figure 6.

Effect of rhBMP-2 on inflammatory interleukin production on microstructured Ti surfaces. MG63 cells were cultured on TCPS or Ti surfaces. At confluence, cells were treated with medium (control) or 40 ng/ml rhBMP-2 for 24 hours. Expression of IL10 (A), IL1 α (B), IL6 (C), IL8 (D), * $p < 0.05$, vs. TCPS; # $p < 0.05$, vs. PT; \$ $p < 0.05$, vs. SLA; % $p < 0.05$, vs. control.

Figure 7.

BMP pathway modulation of inflammatory microenvironment produced by MG63 cells on microstructured Ti surfaces. MG63 cells were cultured on modSLA surfaces. Cells were treated with inhibitors targeting specific BMP signaling pathway components TAB/TAK ((5Z)-7-oxozeaenol), Smad (dorsomorphin), or PKA (H-8) in the presence or absence of 40 ng/ml rhBMP-2. Expression of MSX2 (A), IL10 (B), IL1a (C), IL6 (D), IL8 (E), and IL17 (F) were measured after 12h treatment. * $p < 0.05$, vs. control; # $p < 0.05$, rhBMP-2 treatment vs. untreated cells.

Table 1

Primer sequences used in real-time qPCR analysis.

IL1A	F	TTT GAC GAC GCA CTT GTA GC	NM_000575
	R	GCC ATG AAA TGA CTC CCT CTC	
IL1B	F	TGG CAG AAA GGG AAC AGA AAG G	NM_000576
	R	AAC AAA AGG GCT GGG GAT TGG	
IL6	F	GCA GAA AAC AAC CTG AAC CTT C	NM_000600
	R	ACC TCA AAC TCC AAA AGA CCA G	
IL7	F	TCT TCT TCT GTG CTG GAG ATG	NM_000880
	R	GGA CCT TGT TAT GCT GTT GC	
IL8	F	GAC ATA CTC CCA AAC CTT TCC AC	NM_000584
	R	AAA CCT CTC CAC AAC CCT CTG	
IL10	F	TTA TCT TGT CTC TGG GCT TGG	NM_000572
	R	GAA TGA AGT GGT TGG GGA ATG	
IL17	F	TGG GGC AGA ACG AAC TTT AAG	NM_002190
	R	GGC GAA AAT GGT TAC GAT GTG	
MSX2	F	CCT CTC CCT CTC CAC GAA G	NM_002449.4
	R	TGC CTC CGC CTA CAG AAC	
OCN	F	GTG ACG AGT TGG CTG ACC	NM_199173
	R	TGG AGA GGA GCA GAA CTG G	
GAPDH	F	GCT CTC CAG AAC ATC ATC C	NM_002046.3
	R	TGC TTC ACC ACC TTC TTG	