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Osteogenic Response to BMP-2 of hMSCs Grown on Apatite-Coated Scaffolds

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Abstract

Osteoconductive materials play a critical role in promoting integration with surrounding bone tissue and resultant bone repair *in vivo*. However, the impact of 3D osteoconductive substrates coupled with soluble signals on progenitor cell differentiation is not clear. In this study, we investigated the influence of bone morphogenetic protein-2 (BMP-2) concentration on the osteogenic differentiation of human mesenchymal stem cells (hMSCs) when seeded in carbonated apatite-coated polymer scaffolds. Mineralized scaffolds were more hydrophilic and adsorbed more BMP-2 compared to nonmineralized scaffolds. Changes in alkaline phosphatase (ALP) activity within stimulated hMSCs were dependent on the dose of BMP-2 and the scaffold composition. We detected more cell-secreted calcium on mineralized scaffolds at all time points, and higher BMP-2 concentrations resulted in increased ALP and calcium levels. *RUNX2* and *IBSP* gene expression within hMSCs was affected by both substrate and soluble signals, *SP7* by soluble factors, and *SPARC* by substrate-mediated cues. The present data indicate that a combination of apatite and BMP-2 do not simply enhance the osteogenic response of hMSCs, but act through multiple pathways that may be both substrate- and growth factor-mediated. Thus, multiple signaling strategies will likely be necessary to achieve optimal bone regeneration.

Keywords

scaffold; osteoconductivity; osteoinductive; bone morphogenetic protein; simulated body fluid

INTRODUCTION

Bone possesses superior regenerative properties compared to many other tissues, often utilizing its own wound healing mechanisms for complete repair upon sustaining injury. However, nearly 10–15% of orthopaedic injuries present with complications in the form of delayed healing or non-unions, necessitating the need for outside intervention (Einhorn 1998; Komatsu and Warden 2010). The creation of new bone within a defect is dependent on a complex signaling system derived from both mechanical and soluble osteoinductive cues which ultimately regulates human mesenchymal stem cell (hMSC) differentiation (O'Heireamhoin et al. 2011). Autograft bone, the current gold standard for treatment of nonunion bone defects and critical-sized fractures, often has the most success in effectively balancing these regenerative signals. However, problems such as limited availability, additional surgeries, increased risk of infection, and donor site morbidity have required physicians to consider substitute materials. A variety of natural and synthetic materials including allograft bone, metals, and bioceramics have resulted in favorable outcomes, yet each of these materials suffers from limitations including inadequate bone repair and

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physical properties that are difficult to control (Bauer 2007; Bauer and Muschler 2000; Bauer and Schils 1999; Habraken et al. 2007). The development of polymer/ceramic composite materials as bone substitutes is a promising approach as it enables control over degradation kinetics, mechanical properties, and surface morphologies (Kretlow and Mikos 2007; Porter et al. 2009).

The osteoconductivity of biodegradable polymers such as poly(lactide-co-glycolide) (PLG) can be successfully enhanced through substrate immersion in simulated body fluid (SBF) to create an *ex vivo* carbonated apatite coating similar to that found in native bone (Murphy et al. 2005; Murphy and Mooney 2002). The resulting mineral surface contains both calcium and phosphate ions that are not only capable of nucleating further mineral growth, but can also bind functional growth factors (Dong et al. 2007). Apatite-coated substrata including scaffolds and injectable microspheres have been examined by a number of investigators for their ability to contribute towards bone defect repair (Jongpaiboonkit et al. 2009; Kang et al. 2008; Murphy et al. 2004). Recently we demonstrated a process for fabricating thin films and 3-dimensional scaffolds from mineralized microspheres (termed “premineralized” substrates) and subsequently characterized the ensuing hMSC response (Davis et al. 2009). Our premineralized polymeric materials achieved significant increases in hMSC-secreted calcium over nonmineralized and conventional apatite-coated materials. Despite improvements in the overall *in vitro* osteogenic response associated with premineralized scaffolds, possibly due to increased mineral distribution throughout the scaffold, substrate-mediated cues were not sufficiently potent for robust osteogenesis, which motivates the exploration of their osteogenic potential in tandem with soluble osteoinductive signals.

Bone morphogenetic proteins (BMPs) are potent stimulators of bone growth, capable of inducing *de novo* bone growth at either orthotopic and ectopic sites (Kimelman et al. 2007). BMP-2 and BMP-7 (OP-1) are generally regarded as having the most potential for bone regeneration and are already approved by the FDA for spinal fusion and non-union treatments (Kretlow and Mikos 2007). Both osteoinductive proteins are delivered locally using a collagen sponge, a substrate that fails to provide appropriate control over the rate of presentation to the surrounding tissue to avoid potential bone overgrowth and loss of costly protein. Polymeric materials offer improved control over protein release rates, and moderate success in controlling BMP-2 presentation has been observed *in vivo* by adsorbing the factor onto mineralized surfaces (Autefage et al. 2009; Kang et al. 2008; Liu et al. 2007). Therefore, strategies to promote bone repair likely will benefit from filling a bone defect with an osteoconductive substrate while simultaneously providing a potent osteoinductive signal such as BMP-2. However, the impact of 3D osteoconductive substrates coupled with soluble signals on hMSC cell differentiation remains unclear.

We investigated the influence of BMP-2 in the presence of carbonate apatite on the osteogenic differentiation of hMSCs when seeded in biodegradable PLG scaffolds (Fig. 1). Biomaterialized polymeric scaffolds were fabricated from premineralized microspheres to increase the uniformity of mineral and augment cell-mineral contact throughout the scaffold. This study examines the material properties, BMP-2 binding ability, and the osteogenic differentiation of hMSCs between nonmineralized and premineralized scaffolds in the presence of various doses of this potent osteoinductive protein.

2. MATERIALS AND METHODS

2.1 Materials

Poly(lactide-co-glycolide) pellets (lactide:glycolide = 75:25; inherent viscosity 0.6–0.8, MW=120 kDa) were purchased from Lakeshore Biomaterials (Birmingham, AL, USA). Modified simulated body fluid (mSBF) was prepared as previously described (Davis et al.

2009) and consisted of the following reagents dissolved in distilled H₂O: 141 mM NaCl, 5.0 mM CaCl₂, 4.2 mM NaHCO₃, 4.0 mM KCl, 2.0 mM KH₂PO₄, 1.0 mM MgCl₂, and 0.5 mM MgSO₄. The solution was held at pH 6.8 to avoid homogeneous precipitation of CaP phases. Recombinant human BMP-2 was purchased from PeproTech (Rocky Hill, NJ, USA). All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2 Substrate preparation

PLG microspheres were formed using a standard double emulsion technique (Cohen et al. 1991). For premineralized polymeric substrates, microspheres were hydrolyzed for 10 min in 0.5M NaOH to functionalize the polymer surface, and rinsed in distilled H₂O. Microspheres were immediately placed in mSBF and incubated at 37°C for 7 d, making sure to exchange the solution daily to maintain appropriate ion concentrations. Scaffolds were fabricated using a gas foaming/particulate leaching method as described (Davis et al. 2009). Briefly, 8 mg of polymer microspheres, either nonmineralized or premineralized, and 152 mg of sodium chloride (250–425 µm) were combined and mixed prior to loading into a custom made stainless steel die and compressed at 1500 psi for 1 min using a Carver Laboratory Press to yield solid disks (thickness = 1.5 mm; diameter 8.5 mm). The samples were exposed to high pressure CO₂ gas (800 psi) for 16 h to saturate the polymer with gas. A thermodynamic instability was created by rapidly decreasing the gas pressure to ambient pressure, resulting in the nucleation and growth of CO₂ pores within the polymer matrices. Scaffolds were bisected in cross-section, and the NaCl particles subsequently were removed by leaching the matrices in distilled H₂O for 24 h. All processing steps were performed at ambient temperature.

2.3 Substrate characterization

The porosity measurements were determined by ethanol displacement as previously described (He et al. 2010; Zhang and Ma 1999). The scaffold was placed in a custom-made vacuum bottle and 100% ethanol was used as the displacement liquid, as it was assumed it would not impregnate the PLG material. The scaffold was kept in the static fluid for 5 min and a series of depressurization cycles subsequently followed to ensure fluid penetration into the pores. This process was continued until the discontinuation of bubbles was observed from the scaffold.

Mineral distribution within polymeric scaffolds was qualitatively observed by the adsorption of trypan blue as described (Kim et al. 2007; Kim et al. 2006). Briefly, scaffolds were placed in a 0.4% (w/v) solution of trypan blue (Alfa Aesar, Ward Hill, MA) for 20 s. Immediately after, scaffolds were rinsed in distilled H₂O twice before being placed in 100% EtOH on a shaker. The EtOH was changed every minute for 10 cycles until all unbound dye was removed. Scaffolds were once again rinsed in distilled H₂O and dried prior to observation.

The capabilities of nonmineralized, functionalized (formed from microspheres solely incubated in NaOH) and premineralized scaffolds to bind BMP-2 were measured indirectly by detecting unbound protein in the surrounding fluid. BMP-2 dissolved in distilled H₂O without a carrier protein was fluorescently labeled using a commercially available kit (Dylight Labeling Kit, Pierce Biotechnology) following the manufacturer's instructions. The freshly labeled growth factor was diluted in PBS to obtain a range of concentrations (25, 100, 150, 200 ng/ml). Scaffolds were incubated at 4°C in 300 µl of PBS containing labeled BMP-2 solutions for 4 h. Quantities of BMP-2 that did not adsorb to the scaffold (and thus remained in solution) were determined fluorescently (excitation: 493 nm, emission: 518 nm) with a microplate reader (BIO-TEK Synergy HTTR, Wisnooski, VT). To account for autofluorescence of substrate degradation products, additional scaffolds were incubated in

PBS without growth factor, and these values were subtracted from those observed in the experimental groups. BMP-2 adsorption to the substrate containers was not significant.

2.3 Cell seeding and culture

Human mesenchymal stem cells (hMSCs) were purchased from Lonza (Walkersville, MD, USA) at passage 2 and cultured in DMEM supplemented with 10% fetal bovine serum (JR Scientific, Woodland, CA, USA) and 1% penicillin/streptomycin (Mediatech Inc., Herndon, VA, USA) until use at passage 4–6. Scaffolds were sterilized in 95% ethanol for 30 min followed by two rinses in PBS for 10 min each. 5×10^5 hMSCs in 35 μ L composed of equal volumes of growth factor-reduced Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) and DMEM were statically seeded onto each scaffold and allowed to attach for 1 h. The cell-seeded constructs (n=4 for each group) were then moved to fresh 12-well plates containing 2 ml of DMEM including osteogenic supplements (10 mM β -glycerophosphate, 50 μ g/ml ascorbate-2-phosphate and 10 nM dexamethasone) and BMP-2 (0, 25, 100 or 200 ng/mL). Constructs were cultured in a standard incubator on an XYZ shaker at 25 rpm, and media was changed twice per week.

2.4 Progenitor cell proliferation and differentiation

Substrates were washed twice with PBS and minced with a razor blade before the addition of 500 μ L passive lysis buffer (Promega, Madison, WI). The mixture was sonicated briefly to break up the contents, and then centrifuged at 10,000 rpm for 5 min at 4°C. DNA content in each half-scaffold was subsequently quantitated with the Quant-iT Picogreen dsDNA kit (Invitrogen, Carlsbad, CA). The supernatant was also assayed for alkaline phosphatase (ALP) activity by incubating with 50 mM *p*-nitrophenyl phosphate (PNPP) in an assay buffer (100 mM glycine, 1 mM $MgCl_2$, pH 10.5) at 37°C as previously described (Davis et al. 2009). Absorbance was measured at 405 nm and converted to ALP activity using the extinction coefficient for PNPP ($1.85 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). Cell-secreted calcium on polymeric scaffolds was measured by exposing the remaining solid precipitate to 0.9 N H_2SO_4 for 16 h, followed by centrifugation at 10,000 rpm for 10 min. The supernatant was reacted with cresolphthalein complexone and quantified spectrophotometrically at 570 nm (Cohen and Sideman 1979). The calcium concentration was calculated from a standard curve generated from a serial dilution of a calcium standard solution. Calcium assays were performed on acellular scaffolds and results were subtracted from those obtained from cell-seeded scaffolds to quantify calcium derived from cell-secretion rather than non-specific deposition.

The expression of osteogenic genes was assessed with qPCR at 7 and 21 d for scaffolds supplemented with 100 ng/mL BMP-2, a dosage selected in light of other *in vitro* results and due to its prevalence in previous studies (Jørgensen et al. 2004; Kim et al. 2008; Osyczka et al. 2004; Zhang et al. 2009). At collection, scaffolds were washed with PBS and RNA was collected using the RNeasy Micro Kit (Qiagen, Valencia, CA). 500 ng of total RNA was reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen). qPCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA) on a Mastercycler® Realplex (Eppendorf, Westbury, NY). Primers and TaqMan™ probes for *RUNX2*, *SP7*, *SPARC*, and *IBSP* were purchased from Applied Biosystems (Foster City, CA). Amplification conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Quantitative PCR results were normalized to *TBP* transcript levels to yield ΔC_t . Relative expression was calculated as $2^{-\Delta C_t}$.

2.5 Statistical analysis

Data are presented as mean $\pm$ standard deviation from at least four replicates. The statistical significance was assessed by a two-way ANOVA followed with Bonferroni post-testing

except for Figure 2, which was assessed by a two-tailed unpaired Student's *t*-test and probability values (*p*) for significance were calculated; $p < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad Prism® 4 analysis software (GraphPad Software, San Diego, CA).

3. RESULTS

3.1 Substrate characterization

Substrate porosity remained high with scaffolds formed from apatite-coated microspheres, and no significant differences in porosity were observed between groups (Fig. 2a). This suggests that salt concentration and size, not mineral inclusion, represent the primary contributing factor towards porosity in these substrates.

After incubation and subsequent washings, nonmineralized scaffolds revealed a limited affinity for the hydrophilic trypan blue, while premineralized scaffolds exhibited a blue hue (Fig. 2b). Thus, the wettability of PLG, a hydrophobic polymer, was unchanged by the scaffold formation process and was offset by alkaline treatment and the deposition of carbonated apatite on its surface. Spreading of the dye on premineralized scaffolds was nonuniform, suggestive of inhomogeneities on the surface of premineralized substrates derived from mineral content on changes due to processing.

We determined that BMP-2 had greater affinity for premineralized scaffolds than nonmineralized scaffolds at all protein concentrations when using fluorescently-labeled protein and quantifying the residual fluorescence within the surrounding fluid after incubation (Fig. 3). Interestingly, scaffolds comprised of functionalized PLG microspheres, but not mineral, bound BMP-2 as well as premineralized scaffolds (*data not shown*). Functionalization of polymeric substrates imparts a negative surface charge, thereby mitigating the hydrophobic nature of nonmineralized polymeric scaffolds and its limited affinity for BMP-2.

3.2 Progenitor cell proliferation and differentiation

Independent of BMP-2 dose, there was a trend for increased alkaline phosphatase (ALP) activity from days 0 to 28 (Fig. 4). There was no significant difference in ALP activity between hMSCs cultured on premineralized and nonmineralized scaffolds in osteogenic media supplemented with 0 or 200 ng/mL BMP-2. In contrast, cells on premineralized substrates exhibited significantly increased ALP activity at day 7 in the presence of 25 ng/mL and days 7 and 21 for 100 ng/mL BMP-2. We quantified similar seeding efficiencies on each scaffold type, and no differences were noted between DNA content of cells on either substrate at all time-points and for all BMP-2 concentrations (*data not shown*).

Similar to ALP activity, there was an increase in calcium deposition from days 0 to 28 independent of BMP-2 dose (Fig. 5). At each dose of BMP-2 tested, calcium deposition was significantly higher in pre-mineralized scaffolds compared to nonmineralized scaffolds. We determined a noticeable increase in calcium content for cells on nonmineralized scaffolds only when treated with 200 ng/mL BMP-2.

The expression of mRNA characteristic of osteogenesis was quantified with qPCR at early and late time points (days 7 and 21) for hMSCs seeded on scaffolds and cultured in 100 ng/mL BMP-2 (Fig. 6). This single BMP-2 dosage was selected due to the observed differences in ALP activity between groups. *RUNX2*, the master transcription factor for osteogenesis, decreased for hMSCs in the presence of BMP-2 at day 7 for both substrates, yet mRNA levels were significantly higher for cells in premineralized scaffolds in the presence of BMP-2 compared to the scaffold alone at day 21. Osterix (*SP7*), a *RUNX2* target, was

increased in hMSCs for both premineralized and nonmineralized scaffolds at day 21 in the presence of BMP-2. Compared to cells on premineralized scaffolds and regardless of BMP-2 dosage, cells on nonmineralized scaffolds had significantly more osteonectin (*SPARC*) expression, a matrix protein necessary for calcium nucleation, at both days 7 and 21. Finally, hMSCs grown on premineralized scaffolds in the presence of BMP-2 exhibited significantly greater levels of bone sialoprotein (*IBSP*), another matrix protein, at day 21 compared to cells on all other scaffold groups.

4. Discussion

The balanced design of bioactive materials for guiding bone repair requires a comprehensive understanding of how substrate-mediated cues regulate cell interactions with soluble signals. The deposition of a biomineral on the surface of synthetic polymers yields hybrid biomaterials with enhanced osteoconductivity while maintaining desired biodegradability. Our mineralization process, previously shown to enhance hMSC-secreted mineral (Davis et al. 2009), entails microparticle hydrolysis, subsequent incubation in mSBF, and then scaffold formation, resulting in constructs with biomimetic apatite deposited throughout their volume. BMP-2, a protein known to direct progenitor cells toward the osteoblastic lineage, was supplied in the media for the duration of culture. These data reveal the important interactions between osteoinductive proteins such as BMP-2 with the carbonate apatite on biomineralized polymeric substrates and demonstrate the resulting osteogenic response of hMSCs in an environment designed to enhance bone formation.

Construct microarchitecture is a key mediator in progenitor cell differentiation. Several studies have reported the role of pore size and porosity on inducing hMSCs towards the osteoblastic phenotype (Mygind et al. 2007; Whang et al. 1999). Moreover, changes in scaffold porosity led to alterations in growth factor binding and cell response to BMP-2 (Tsuruga et al. 1997; Whang et al. 2000). In both nonmineralized and premineralized constructs, salt crystals of a defined size (250–425 μm) were used to create substrates with high porosities, and hence pore size should be relatively similar. The addition of hydroxyapatite nanoparticles to similar scaffolds markedly decreased porosity (He et al. 2010). Thus, to characterize potential differences in porosity for these two substrates, we employed a volume displacement method that ultimately demonstrated similarly high porosities for both nonmineralized and premineralized scaffolds. The alkaline treatment employed to hydrolyze the polymer increases the surface area and generates nanotopographical changes on the polymer surface previously shown to increase osteoblast adhesion (Kay et al. 2002; Smith et al. 2007). We previously observed increased osteogenic differentiation of hMSCs on premineralized scaffolds compared to nonmineralized, alkaline-treated substrates with similar levels of cellularity (Davis et al. 2009), indicating that NaOH-induced nanotopographical changes have a less pronounced effect on cell adhesion and differentiation in three-dimensions. However, the surface of these scaffolds is a combination of carbonated apatite and NaOH-treated domains, as mineral coverage is not completely uniform. The increased surface area produced by alkaline treatment prior to biomineralization may provide additional sites for BMP-2 binding and adsorption to the substrate, and this surface heterogeneity may contribute to these data. Osteogenic differentiation of hMSCs is also affected by differences in substrate stiffness (Engler et al. 2006). Our previous study demonstrated no differences in compressive moduli between premineralized and nonmineralized substrates (Davis et al. 2009).

In light of similar porosities and compressive moduli between substrates, differences in the expression of osteogenic markers by hMSCs seeded on premineralized and nonmineralized scaffolds are likely attributed to the combinatorial effects of soluble signals with other material properties such as wettability or increased surface area following microsphere

functionalization that result in more protein binding sites. Premineralized scaffolds adsorbed more trypan blue compared to the relatively hydrophobic nonmineralized scaffolds and are likely more capable of adsorbing surrounding plasma proteins. Indeed, when specific adsorption of BMP-2 to the substrates was investigated, premineralized scaffolds adsorbed greater amounts of protein compared to nonmineralized substrates for all BMP-2 concentrations tested. Interestingly, we observed that functionalized scaffolds adsorbed similar amounts of BMP-2 as premineralized scaffolds. Functionalization of PLG scaffolds creates hydrolyzed, carboxylic acid rich surfaces. Although the predominant mechanism for BMP-2 adsorption to apatite surfaces is the interaction between BMP's carboxylic group with surface calcium ions, functionalization of PLG likely lowers the polymer's inherent hydrophobicity, hence creating more water-bridged hydrogen bonds (Dong et al. 2007). Thus, functionalized polymers may offer another approach to increase BMP-2 binding without necessitating the presence for mineral.

In agreement with earlier studies, ALP activity of hMSCs was not increased when seeded on mineralized substrates versus nonmineralized scaffolds in the absence of BMP-2 (Davis et al. 2009; Murphy et al. 2005). However, the addition of 25 and 100 ng/mL BMP-2 to cells on premineralized substrates resulted in greater activities at certain time points, suggesting that substrate-mediated cues acted synergistically with the soluble signals at these concentrations. At 200 ng/mL BMP-2, differences in the cellular response as a function of the substrate were masked and the magnitude of ALP activity was increased for both groups, suggesting that BMP-2 became the principal mediator in progenitor differentiation. At all BMP-2 concentrations, cell-secreted calcium for hMSCs seeded on premineralized scaffolds was significantly greater than for cells cultured on nonmineralized scaffolds, suggesting that substrate composition was of more importance. At 200 ng/mL, these differences were not apparent at early time points, and it was only at this concentration that an increase in calcium deposition was observed for cells on nonmineralized substrates. These results suggest that premineralized scaffolds nucleate cell-secreted calcium more efficiently than nonmineralized substrates. Moreover, these data confirm that higher concentrations of BMP-2 can override the contribution of substrate-mediated cues, and hMSC osteogenic differentiation may not benefit from substrate mineralization at such protein levels.

Construct composition, in conjunction with a soluble osteoinductive factor, had a considerable effect on osteogenic gene expression profiles. These studies were performed with a single BMP-2 concentration to further analyze apparent differences in ALP activity among groups while using a dosage that is commonly cited in the literature as effective for inducing hMSC osteogenic differentiation. Our data demonstrate down-regulation of *RUNX2* with the addition of BMP-2 at an early time point (although not significant for premineralized substrates) and a significant increase in expression for only premineralized substrates at a late time point, suggesting that *RUNX2* expression is both mineral- and BMP-2 dependent. However, osterix (*SP7*) expression exhibited only significant increases in expression for substrates cultured in the presence of BMP-2 with comparative results between premineralized and nonmineralized groups, suggesting that BMP-2 is the predominant osteogenic factor. Osterix is generally considered to be downstream of *RUNX2* in the progenitor osteogenic differentiation pathway (Nakashima et al. 2002), and thus, it was surprising to see a large increase in *RUNX2* expression at day 21. Others have reported that osterix expression does not consistently follow the same trend as *RUNX2*, as there are other pathways that act alongside, or independent of, *RUNX2* to modulate osterix expression during osteogenesis (Celil et al. 2005). Osteonectin (*SPARC*) exhibited poor correlation with the presence of the osteoinductive protein. Although hMSCs on nonmineralized substrates exhibited significantly more expression at both time points, cells on each substrate failed to upregulate gene expression in response to the addition of BMP-2. Osteonectin is a glycoprotein expressed in bone undergoing active remodeling. Since premineralized

substrates already possessed mineral nucleation sites due to mSBF incubation, it is possible that hMSCs seeded on nonmineralized scaffolds had more osteonectin gene expression as they attempted to remodel the polymer scaffold to nucleate mineral. Bone sialoprotein (*IBSP*) expression was dependent on both substrate cues and signal induction, as cells on premineralized substrates in the presence of BMP-2 demonstrated significantly increased gene expression at day 21. hMSCs exposed to large amounts of apatite demonstrate increased deposition of bone sialoprotein in culture (Bhumiratana et al.). The addition of BMP-2 in the presence of mineral might possibly alter the timing of bone sialoprotein expression, resulting in a peak earlier in the culture period. Furthermore, premineralized and nonmineralized scaffolds have distinct affinities for BMP-2, potentially enabling differences in protein adsorption and presentation to hMSCs. However, protein affinity for both scaffolds is a dynamic variable given the increasing calcium deposition on the substrate. This potential contribution to these results merits further investigation.

The present data indicate that the combination of apatite and BMP-2 do not simply enhance the osteogenic response of hMSCs, but act through multiple pathways that may be both substrate- and growth factor-mediated. BMP-2 is a costly recombinant growth factor, and bone regeneration strategies should attempt to limit its use to therapeutic doses to control patient costs. While many have attempted to present BMP-2 from osteoconductive substrates containing bone-like minerals as a means to enhance osteogenesis, the combination of soluble signals in conjunction with substrate-mediated cues remains largely unexplored. These results suggest that the osteogenic differentiation of hMSCs is dependent on both substrate- and soluble-mediated cues, although one does not simply augment the other. Each has a more potent effect on different aspects of progenitor differentiation, and the magnitudes of these effects are dependent on BMP-2 concentration. In light of these observations, multiple signaling strategies may be necessary to achieve optimal bone regeneration.

5. Conclusion

This study investigated the influence of BMP-2 in the presence of carbonated apatite on the osteogenic differentiation of hMSCs when seeded in nonmineralized and premineralized substrates. Premineralized scaffolds possessed a more hydrophilic surface and thus adsorbed more BMP-2 compared to nonmineralized substrates. However, markers for cell differentiation were influenced by substrate and soluble signals in both independent and co-dependent paradigms; the conjunction of substrate-mediated cues and soluble signals did not synergistically augment cell osteogenic drive.

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Figure 1.
Flow chart of scaffold fabrication and experimental design.

Figure 2.
A) Scaffold porosity (chart values represent mean $\pm$ SD; n=4). B) Trypan blue staining of nonmineralized and premineralized scaffolds.

Figure 3. Residual fluorescently labeled BMP-2 remaining in solution after scaffold incubation for 4 h. Larger RFU values indicate decreased binding to substrate. Data represent mean $\pm$ SD; $n=3$; * $p < 0.05$ vs. premineralized and functionalized scaffolds.

Figure 4.

Alkaline phosphatase activity as a function of scaffold composition and supplemental BMP-2 concentrations: A) 0 ng/mL, B) 25 ng/mL C) 100, and D) 200 ng/mL. Chart values represent mean $\pm$ SD; $n=4$; * $p < 0.05$ vs. nonmineralized scaffolds.

Figure 5. Calcium content of premineralized and nonmineralized scaffolds at A) 0 ng/mL, B) 25 ng/mL, C) 100 ng/mL, and D) 200 ng/mL supplemental BMP-2 concentrations. Chart values represent mean $\pm$ SD; n=4; * p <0.05 premineralized relative to nonmineralized scaffolds.

Figure 6.

Quantitative PCR was used to probe osteogenic gene expression by hMSCs on premineralized and nonmineralized scaffolds at a supplemental 100 ng/mL BMP-2 concentration. A) *RUNX2*, B) *SP7*, C) *SPARC*, and D) *IBSP* expression normalized to *RPL13* expression. Values are mean $\pm$ SD; n=4; * p <0.05 vs. non-BMP-2 counterpart; # p <0.05 vs. premineralized counterpart.