Biochemical and Biophysical Research Communications 401 (2010) 20–25

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Bone morphogenetic protein 6 drives both osteogenesis and chondrogenesis in murine adipose-derived mesenchymal cells depending on culture conditions

Carly M. Kemmis, Ali Vahdati, Holly E. Weiss, Diane R. Wagner *

Department of Mechanical Engineering and Bioengineering Graduate Program, University of Notre Dame, Notre Dame, IN 46556, USA

ARTICLE INFO

Article history:
Received 19 August 2010
Available online 9 September 2010

Keywords:
Osteogenesis
Chondrogenesis
Adipose-derived mesenchymal stem cells
BMP-6
Culture conditions

ABSTRACT

Bone morphogenetic proteins (BMPs) play a dual role as a factor in both bone and cartilage development and correspondingly have the therapeutic potential to regenerate both tissues. Given this dual nature, previous *in vitro* research using BMPs has relied on distinct media formulations and culture conditions to drive undifferentiated cells to the osteogenic or chondrogenic lineage. To isolate the impact of culture conditions and to explore the effect of BMP-6 on murine adipose-derived mesenchymal cells (ASCs), ASCs were seeded in either monolayer or pellets in an identical medium containing BMP-6. Results indicate that BMP-6 differentially promotes osteogenesis and chondrogenesis in ASCs depending on culture conditions. BMP-6 potently induced alkaline phosphatase activity and mineralization in ASCs cultured in monolayer conditions. In contrast, BMP-6 enhanced proteoglycan accumulation in ASCs seeded in chondrogenic pellet culture. A comparison of gene expression suggests that the differentiating effect of BMP-6 is specific to the particular culture condition. This study highlights the importance of the interactions between chemical signaling and microenvironmental cues in directing cell fate.

1. Introduction

Bone morphogenetic proteins (BMPs) are signaling molecules in the transforming growth factor-beta (TGF-β) superfamily that play significant roles in both bone and cartilage morphogenesis [1] and are also effective in bone and cartilage repair [2–4]. BMP-2 and BMP-7 are clinically available to enhance and accelerate bone repair and formation (INFUSE®, Medtronic, Sofamor Danek, Memphis, TN; OP-1 Implant® and OP-1 Putty®, Stryker, Kalamazoo, MI). Additionally, *in vitro* studies demonstrate the multi-potency of BMP-2, BMP-6 and BMP-7 in osteogenic and chondrogenic differentiation of marrow-derived mesenchymal stromal cells (MSCs) for tissue engineering and regenerative applications [5–10]. However, the dual nature of BMPs is not well understood and *in vitro* models to study the switch between the osteogenic and chondrogenic lineages have yet to be developed. *In vitro* investigation into the multipotent effects of BMPs relies on specific culture and media conditions to drive undifferentiated cells to the selected lineage, making it difficult to isolate factors that mediate a switch in BMP-induced lineage commitment. Thus, development of a single medium that harnesses the bipotent effect of BMPs to induce both osteogenesis and chondrogenesis could advance the understanding of the role of the cell culture environment in driving cell fate.

Efficient *in vitro* osteogenesis and chondrogenesis depends on culture conditions, and previous studies indicate that several features of the cellular microenvironment are critical for commitment to these lineages. For example, integrin attachment, focal adhesion formation and subsequent cell signaling drives osteogenic differentiation in both monolayer culture and three-dimensional scaffolds [11–13]. Furthermore, enhanced osteogenic differentiation of marrow-derived mesenchymal stromal cells (MSCs) cultured on stiff substrates or at low densities indicate cytoskeletal tension and a spread cell shape may also contribute to early determination of cell fate [14,15]. In contrast, culture conditions maintaining a rounded cell shape such as in alginate and agarose gels, high density micromass culture, and three dimensional pellet culture are favorable for chondrogenesis, suggesting that commitment to the chondrogenic lineage may be dependent on cell shape [16–19]. Furthermore, cell-to-cell interactions and integrin-mediated adhesions to newly formed extracellular matrices may also modulate chondrogenic differentiation [20–23].

In addition to implementing specific culture conditions to induce osteogenesis and chondrogenesis, classical differentiation media for these lineages have distinct formulations. However, unlike culture conditions, media formulations vary greatly and significant overlap can be found between osteogenic and chondrogenic differentiation media. For example, both osteogenic and chondrogenic media may contain BMPs to enhance differentiation

* Corresponding author. Address: Aerospace and Mechanical Engineering, 145 Multidisciplinary Research Building, Notre Dame, IN 46556, USA. Fax: +1 574 631 2144.

E-mail addresses: ckemmis@gmail.com (C.M. Kemmis), avahdati@nd.edu (A. Vahdati), hweiss1@nd.edu (H.E. Weiss), dwagner@nd.edu (D.R. Wagner).

URL: http://www.nd.edu/~dwagner (D.R. Wagner).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.08.135

to either lineage. Additionally, osteogenic media commonly contain serum while chondrogenic media do not, but differentiation to either lineage may be successfully induced in either case.

In light of these facts, we hypothesized that a single medium containing BMPs could induce both osteogenesis and chondrogenesis depending on the cell culture environment. To address this, we investigated the effect of BMP-6 on murine adipose-derived mesenchymal cells (ASCs) in both osteogenic and chondrogenic culture conditions. ASCs are a promising cell source for tissue engineering applications due to their accessibility, abundance and multi-lineage potential; many studies have demonstrated that ASCs have the capacity to differentiate into several tissues, including bone and cartilage [24]. Although the osteogenic effect of BMP-6 on ASCs has not been previously reported, Estes et al. successfully achieved chondrogenic differentiation in ASCs seeded in alginate beads and cultured in medium containing BMP-6 [25]. Furthermore, the dual role of BMP-6 as a factor in both osteogenesis and chondrogenesis has been demonstrated in MSCs using distinct media and culture conditions [6,8].

In this study, we seeded murine ASCs in either monolayer or pellets and induced differentiation with a single medium to investigate the impact of BMP-6 and the microenvironment facilitated by culture conditions on osteochondral differentiation. Here, we provide strong evidence that BMP-6 plays a dual role in differentiation of murine ASCs and demonstrate that the microenvironment found in monolayer and pellet culture may influence the specificity of BMP-6.

2. Methods

ASCs were isolated from male FVB mice at 3–4 weeks of age in accordance with the Notre Dame Animal Care and Use Committee approved protocol as has been described previously [26]. Cells were expanded in growth medium consisting of high glucose DMEM (Mediatech, Manassas, Virginia), 10% heat-inactivated fetal bovine serum (Omega Scientific, Tarzana, California), and 100 IU Penicillin/100 μg/mL Streptomycin (Mediatech). Cells were expanded up to two passages in a humidified incubator at 37 °C and 5% CO_2 before being seeded for experiments.

For monolayer culture, ASCs were seeded at 1300 cells/cm^2 in growth medium and allowed to adhere. Alternatively, ASCs were pelleted by centrifugation at 200,000 cells/well in sterile polypropylene 96-well plates. Differentiation medium was applied to the cells 4 h after seeding, consisting of growth medium supplemented with 100 μg/mL ascorbic acid (Wako, Richmond, Virginia) and 10 mM β-glycerophosphate (Sigma–Aldrich, St. Louis, Missouri) and, when applicable, 100 ng/mL BMP-6 (R&D Systems, Minneapolis, Minnesota) and was replenished every 3–4 days.

To assess proliferation, total DNA content of ASCs seeded in monolayer or pellet was assessed at 0, 3, 7 and 10 days with the Quant-iT PicoGreen dsDNA Reagent Kit (Invitrogen, Carlsbad, California), per manufacturer's instructions. For each timepoint, cell proliferation was expressed as fold change from day 0, $n = 3$.

To evaluate early osteogenic differentiation, alkaline phosphatase activity was quantified in ASCs cultured in monolayer with the colorimetric SensoLyte pNPP Alkaline Phosphatase Assay Kit (Anaspec, San Jose, California) after 7 days using a modified protocol. Alkaline phosphatase activity of the supernatant was measured according to manufacturer's instructions and normalized to total protein concentration via BCA Assay (Pierce, Rockford, Illinois), $n = 4$. Alternatively, cells were fixed and stained with Fast Blue as previously described [26]. ASCs grown in monolayer for 14 days were fixed and stained 0.2% Alizarin red as a late marker of osteogenic differentiation as previously described [26]. Alizarin stain was resuspended with 100 mM hexadecylpiridinium chloride (Sigma–Aldrich) and the absorbance measured at 560 nm using a Victor 3 microplate reader (Perkin Elmer, Waltham, Massachusetts), $n = 6$.

ASCs cultured in pellets were grown for 12 days, at which time sGAG accumulation was quantified. For each sample, eight pellets were pooled, washed with PBS and incubated overnight with 20 μg/mL papain digestion buffer at 58 °C. Samples were briefly vortexed, boiled for 5 min, centrifuged at 12,000*g* for 15 min and the pellet reserved for DNA quantification. The supernatant was collected and proteoglycan content determined via WeisLab sGAG quantitative kit (Alpco, Salem, New Hampshire). The pellet was briefly boiled with 0.02% SDS and incubated overnight with125 μg/mL Proteinase K (Sigma–Aldrich) at 58 °C. DNA was quantified via Quant-iT PicoGreen dsDNA kit and used to normalize sGAG content, $n = 3$. Pellets differentiated for 14 days were washed twice with PBS and fixed for 15 min in 4% PFA and 4% sucrose in PBS. Following fixation, pellets were rinsed in PBS and embedded in optimal cutting temperature compound. 10 μm sections were stained with Alcian Blue Solution and Fast Red Nuclear Stain. The sections were imaged using a Nikon ME600 microscope.

Total RNA was isolated using RNeasy kit (Qiagen, Valencia, California) according to manufacturer's instructions after 3, 7 and 10 days for cells cultured in monolayer or 7, 14, and 21 days for pellet culture, $n = 3$. 1 μg cDNA stocks for each sample were synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California). cDNA stocks were analyzed in triplicate (300 ng/well) for gene expression via Fast SYBR Green Mastermix (Applied Biosystems). Data with primers sets specific for *Runx2* (Forward: CGGCCCTCCCTGAACTCT; Reverse: TGCCTGCCTGGGATCTGTA), *Opn* (Forward:AGCAAGAAACTCTTCCA AGCAA; Reverse: *GTGAGATTCGTCAGATTCATCCG*), *Ocn* (Forward: CTGACCTCACAGATCCCAAGC; Reverse: *TGGTCTGATAGCTCGTCACAAG)*, *Agc* (Forward: CTGGGATCTACCGCTGTGAAG; Reverse: *TGTGGAAA-TAGCTCTGTAGTGGA*), *Sox9* (Forward: TTCCTCCTCCGGCATGAGTG; Reverse: CAACTTTGCCAGCTTGCACG), *Col2a1* (Forward: TGGCTGG AGGGTATGACGAG; Reverse: *TTGCCTTGAAATCCTTGAGGG*), were normalized against *18S* (Forward: *AGTCCCTGCCCTTTGTACACA;* Reverse: GATCCGAGGGCCTCACTAAAC). Data were analyzed via $2^{-\Delta\Delta CT}$ method using monolayer or pellet control as a reference for osteogenic and chondrogenic genes, respectively.

Where applicable, statistical significance was determined by one- or two-way ANOVA and Bonferroni's or Tukey's post-test ($p < 0.05$) using GraphPad Prism Software (La Jolla, California).

3. Results

To begin exploration into the roles of culture conditions and BMP-6 during ASC differentiation, we first established the effect of BMP-6 on cell proliferation in both monolayer and pellet cultures, as determined by DNA content (Fig. 1). ASCs seeded in monolayer attached to the culture dish and experienced positive cell growth over the course of 10 days. In contrast, ASCs seeded

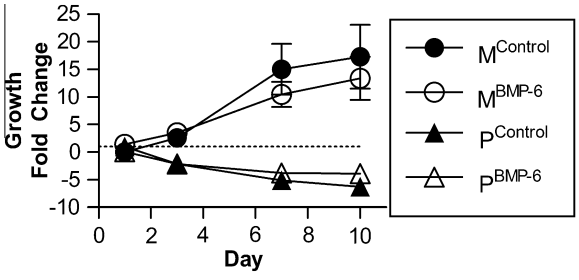

Fig. 1. AMCs were seeded in monolayer or pellets and differentiated with 0 or 100 ng/ml BMP-6. Cell proliferation was determined by assessing DNA content and reported as fold change from day 0, $n = 3$.

in pellets aggregated to form spherical masses and a decrease in cell number was observed, as previously demonstrated in similar experiments [26,27]. After monolayer culture for 10 days, total DNA content of ASCs supplemented with BMP-6 was slightly lower in monolayer as compared to control. On the other hand, BMP-6 was protective of the viability of cells cultured in pellets.

To determine the action of BMP-6 on osteogenesis of ASCs, gene expression was evaluated of cells seeded cells in monolayer and exposed to 0 or 100 ng/mL BMP-6. At day 3, *Runx2* mRNA expression was upregulated in AMCs cultured in media containing BMP-6 in comparison to control (Fig. 2A). Expression of *Opn* and *Ocn* were strongly induced in a time dependent manner following culture with BMP-6 (Fig. 2B and C). Physical markers of osteogenic differentiation were assessed following culture with either control medium or a medium containing 50, 100 or 250 ng/mL BMP-6. Alkaline phosphatase activity was significantly increased in a dose-dependent fashion when ASCs were cultured in media containing BMP-6 (Fig. 2D and F). BMP-6 also stimulated mineralization of ASCs, as made evident by Alizarin red S quantification and representative staining (Fig. 2E and G).

For chondrogenic differentiation, ASCs were seeded at high density in round bottom 96-well polypropylene plates and centrifuged; spherical pellets naturally formed after several hours. To assess the effects of BMP-6 on cartilage matrix accumulation, the pellets were cultured in control medium or a medium containing 100 ng/mL BMP-6 and chondrogenic gene expression analyzed. The expression of *Sox9* appeared to peak by day 7 in both conditions and was upregulated by BMP-6 on day 7 in comparison to controls (Fig. 4A). In a time dependent upregulation, robust *Agc* (Fig. 4B) and *Col2* (Fig. 4C) expression was observed following culture with BMP-6. An assay quantifying proteoglycans in cell pellets demonstrated increased sGAG accumulation when cells were cultured with BMP-6 (Fig. 3D). This evidence is supported by histological staining of frozen sections with Alcian blue counterstained with Fast Red; pellets cultured with BMP-6 exhibited increased proteoglycan staining compared to control (Fig 3E).

To establish that culture conditions directly influence the effect of BMP-6 on lineage commitment, gene expression from both conditions with 0 or 100 ng/ml BMP-6 was analyzed and compared at day 7. *Runx2* and *Ocn* mRNA expression was strongly upregulated by BMP-6 in monolayer but not in pellet culture (Fig. 4A and B); moreover, basal *Ocn* expression was noticeably decreased in ASCs when cultured in pellets relative to monolayer. Analysis of the early chondrogenic gene, *Sox9*, demonstrates equivalent basal expression and equal upregulation by BMP-6 in both pellet and monolayer conditions (Fig 4C). In contrast, *Agc* expression was markedly decreased when cells are cultured in monolayer, while robust BMP-6-induced upregulation of *Agc* was observed in pellet culture (Fig. 4D). These analyses suggest that monolayer and pellet culture conditions influence basal gene expression and bias the lineage commitment of ASCs toward osteogenic and chondrogenic differentiation, respectively, while application of BMP-6 enhances both osteogenesis and chondrogenesis in a manner consistent with the culture conditions.

4. Discussion

A single medium that drives both osteogenic and chondrogenic differentiation is critical for understanding the role of the culture microenvironment in determining lineage commitment. Therefore, we investigated the effects of BMP-6 on differentiation of murine ASCs to verify our original hypothesis: that a single medium could induce both osteogenesis and chondrogenesis depending on culture conditions. We demonstrated that BMP-6 drives both osteogenic and chondrogenic differentiation of murine ASCs and illustrated the pivotal role culture conditions play in directing BMP-6-induced lineage commitment. When cultured in the medium formulation containing BMP-6, ASCs seeded in monolayer exhibited robust osteogenic gene expression and mineralization while the same cells differentiated in pellets showed evidence of chondrogenic gene expression and extensive proteoglycan accumulation. These observations suggest a dual role for BMP-6 as a factor in both osteogenesis and chondrogenesis.

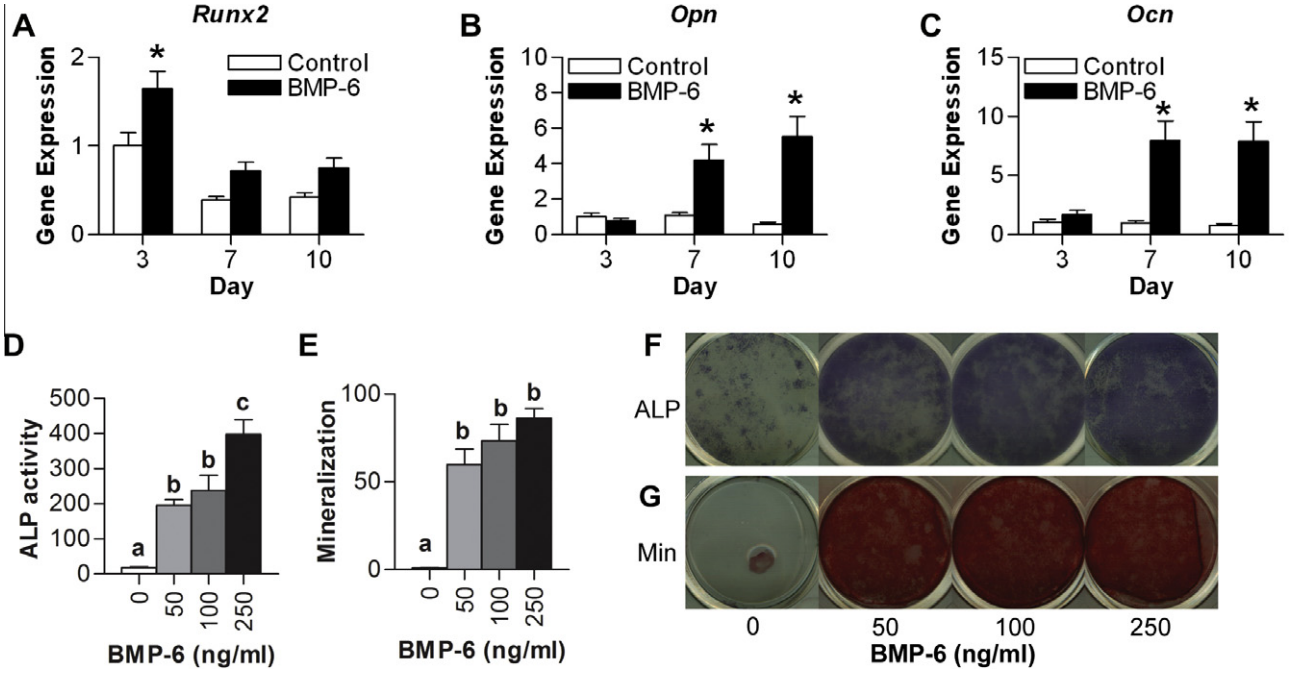

Fig. 2. AMCs seeded in monolayer were differentiated with 0 or 100 ng/ml BMP-6 and RNA isolated for gene expression analysis at days 3, 7 and 10. Expression of (A) *Runx2*, (B) *Opn* and (C) *Ocn* was determined via $2^{-\Delta\Delta Ct}$ method using day 3 control as a reference. *Indicates significant difference from respective time-point control, two-way ANOVA, Bonferroni post-test, $p < 0.05$, $n = 3$. Cells in monolayer were differentiated in medium containing 0, 50, 100 or 250 ng/ml BMP-6. (D) ALP was quantified at day 7 ($n = 4$), (E) subsequent mineralization was quantified at day 14 ($n = 6$) and (F) representative ALP activity and (G) mineralization (Min) were stained with fast blue or Alizarin, respectively. Differing letters indicates significance, one-way ANOVA, Tukey post-test, $p < 0.05$.

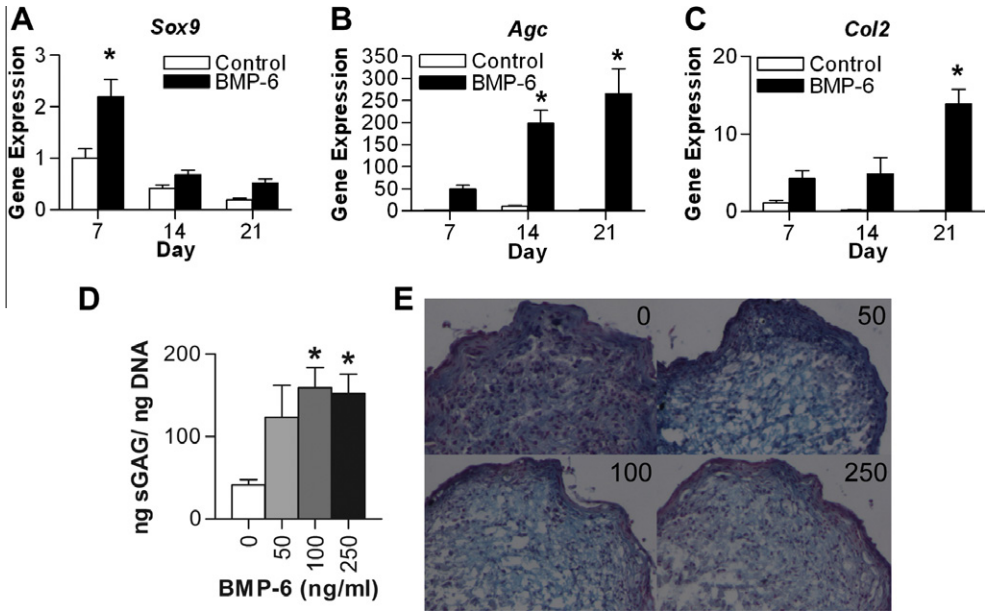

Fig. 3. AMCs seeded in pellet were differentiated with 0 or 100 ng/ml BMP-6 and RNA isolated for gene expression analysis at days 7, 14, and 21. Expression of (A) *Sox9*, (B) *Agc* and (C) *Col2* was determined via $2^{-\Delta\Delta Ct}$ method using day 7 control as a reference. *Indicates significant difference from respective time-point control, two-way ANOVA, Bonferroni post-test, $p < 0.05$, $n = 3$. Cells seeded in pellets were differentiated in medium containing 0, 50, 100 or 250 ng/ml BMP-6 for 14 days. (D) sulfated glycosaminoglycan accumulation was quantified and normalized to DNA content and (E) sections were stained with Alcian blue and fast red (50×). *Indicates significance from control, one-way ANOVA, Bonferroni post-test, $p < 0.05$, $n = 3$.

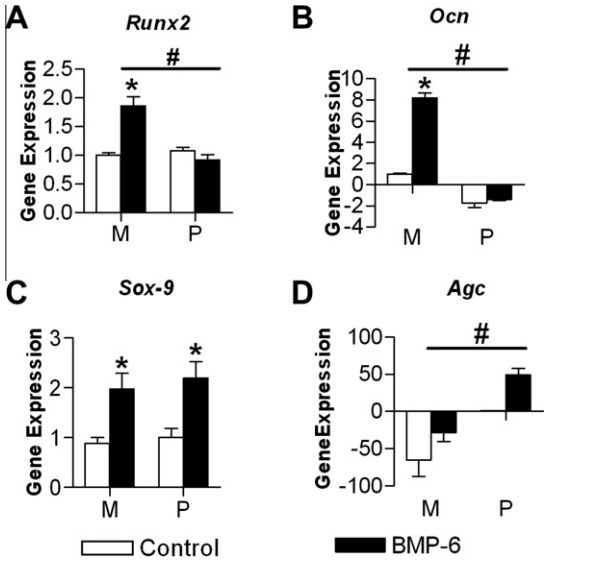

Fig. 4. AMCs seeded in monolayer and pellet were differentiated with 0 or 100 ng/ml BMP-6. RNA was isolated at day 7 and expression of (A) *Runx2*, (B) *Ocn*, (C) *Sox9* and (D) *Agc* was determined via $2^{-\Delta\Delta Ct}$ method using the monolayer control (A–B) or pellet control (C–D) as a reference. #Indicates a difference between culture conditions, two-way ANOVA, $p < 0.05$. *Indicates significant difference from respective control, Bonferroni post-test, $p < 0.05$, $n = 3$.

The observed osteogenic effects of BMP-6 on murine ASCs in monolayer are in agreement with previous reports indicating that BMP-6 has the capacity to drive osteogenesis in MSCs [8]. Although BMPs have a known osteogenic effect in many cell types, prior studies examining their influence on ASCs have been limited. The most notable demonstration that BMPs are effective in driving osteogenesis is the enhanced bone formation observed in both *in vitro* and *in vivo* applications of cells derived from human lipoaspirates expressing adenovirus-mediated rhBMP-2 [28,29]. Furthermore, the osteogenic effect of BMP-2 has been established in murine ASCs *in vitro* [30]. In this previous study, osteogenesis was demonstrated only when BMP-2 was combined with upregulation of BMP receptor IB by retinoic acid, implicating a role for additional factors in BMP-mediated osteogenesis of ASCs [30]. In contrast, the current results demonstrated that the addition of BMP-6 alone induced osteogenic gene expression and robust matrix mineralization of murine ASCs. While application of BMPs in ASC culture has yet to be optimized, BMP-6 is a promising chemical signal that may be effective in directing the osteogenic lineage commitment of ASCs.

Although BMP-6 significantly contributes to both osteogenic and chondrogenic differentiation of ASCs, our study highlights the critical role for culture conditions in determination of cell fate and cellular response to BMP-6. In control medium, the elevation of osteogenic and chondrogenic markers were specific to distinct culture conditions and suggests that culture conditions alone can predispose cells to the osteogenic or chondrogenic lineage. Moreover, BMP-6 enhancement of osteogenic and chondrogenic gene expression was specific to and synergistic with culture conditions, suggesting that microenvironmental cues not only influence lineage commitment but also modulate the cellular response to chemical signals. This work underscores the importance of understanding the role of microenvironmental cues in determining and directing cell fate.

The observed impact of microenvironmental cues on lineage commitment was made possible by a novel medium formulation capable of driving both the osteogenic and chondrogenic differentiation of ASCs. Although previous efforts to develop bipotent media for MSCs have been reported, few have achieved this goal. Perhaps the most successful bipotent medium contained both osteogenic and adipogenic factors and was used to demonstrate that cell density and subsequent changes in cytoskeletal tension influence osteogenic and adipogenic cell fate of human MSCs [14]. To simultaneously drive osteogenesis and chondrogenesis, Guo, et al. designed a bilayer scaffold supporting osteochondral differentiation using a single medium, though it was insufficient in

driving lineage commitment and required pre-culture of MSCs in a separate medium to achieve osteogenesis [31]. Recently, a minimal common medium for osteochondral differentiation of MSCs was reported, but the medium was supplemented with distinct growth factors for osteogenesis and chondrogenesis [32]. Thus, the current study is the first to date to demonstrate osteochondral differentiation using a single bipotent medium.

Our work demonstrates the bipotent, culture specific nature of BMP-6 in driving osteogenic and chondrogenic differentiation of murine AMCs. Despite this striking effect on differentiation, we observed BMP-6-induced expression of *Sox9* in both monolayer and pellet culture conditions. The transcription factor SOX9 is required for chondrogenesis and directly regulates a number of chondrogenic genes, so equivalent *Sox9* gene expression in both culture conditions was unexpected [33–35]. However, *Sox9* alone does not control the switch in commitment to a chondrogenic lineage, as it is expressed in both osteo- and chondroprogenitor cells in development and is upregulated by BMP-2 in osteoprogenitor cells *in vitro* [36,37]. Instead, the ratio of *Sox9* to the osteogenic transcription factor *Runx2* appears to determine osteo-chondro cell fate; high expression of *Sox9* relative to *Runx2* drives chondrogenesis, while high expression of *Runx2* relative to *Sox9* triggers osteogenesis [37,38]. In the current study, *Sox9* expression was equivalent in both culture conditions while *Runx2* was upregulated by BMP-6 only in monolayer, suggesting that regulation of *Runx2* drives the cell fate decision in our system. The interrelationship between *Sox9* and *Runx2* may not be as straightforward as a simple ratio between the two, however, as *Sox9* has been shown to inhibit Runx2 function during skeletogenesis [39].

Future studies that identify the mechanisms responsible for the cell fate decision and elucidate the signaling pathways active in the cell during osteogenic and chondrogenic differentiation are essential to clinical applications of BMP-6 for bone and cartilage therapies. Once these processes are fully understood, a bipotent medium may provide a solution for osteochondral tissue engineering applications requiring simultaneous differentiation of mesenchymal cells into bone and cartilage in a single scaffold.

5. Conclusions

We have assessed a single medium containing BMP-6 for both osteogenic and chondrogenic differentiation of murine AMCs. We have provided strong evidence that the osteogenic and chondrogenic capacity of BMP-6 depends on the microenvironment facilitated by cell culture conditions. Although culture conditions have long been known to influence differentiation of progenitor cells, this work suggests that the switch in BMP-6-induced lineage commitment is determined primarily by conditions present in monolayer or pellet culture and implies that BMP signaling may have multiple interpretations that depend on the culture microenvironment. Future studies that identify the mechanisms responsible for the cell fate decision and elucidate the signaling pathways active in the cell during osteogenic and chondrogenic differentiation are essential to clinical applications of BMP-6 for bone and cartilage therapies.

Acknowledgments

This study was funded by the University of Notre Dame Adult Stem Cell Initiative and the US Army Medical Research and Materiel Command W81XWH-07-0662.

References

[1] B.L. Hogan, Bone morphogenetic proteins: multifunctional regulators of vertebrate development, Genes Dev. 10 (1996) 1580.
[2] R.S. Sellers, D. Peluso, E.A. Morris, The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the healing of full-thickness defects of articular cartilage, J. Bone Joint Surg. Am. 79 (1997).
[3] L. Lin, C. Zhou, X. Wei, et al., Articular cartilage repair using dedifferentiated articular chondrocytes and bone morphogenetic protein 4 in a rabbit model of articular cartilage defects, Arthritis Rheum. 58 (2008) 1067.
[4] R. Kuroda, A. Usas, S. Kubo, et al., Cartilage repair using bone morphogenetic protein 4 and muscle-derived stem cells, Arthritis Rheum. 54 (2006).
[5] K. Hanada, J.E. Dennis, A.I. Caplan, Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells, J. Bone Miner. Res. 12 (1997) 1606–1614.
[6] I. Sekiya, D.C. Colter, D.J. Prockop, BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells, Biochem. Biophys. Res. Commun. 284 (2001) 411–418.
[7] W.S. Toh, H. Liu, B.C. Heng, et al., Combined effects of TGFβ1 and BMP2 in serum-free chondrogenic differentiation of mesenchymal stem cells induced hyaline-like cartilage formation, Growth Factors 23 (2005) 313–321.
[8] M.S. Friedman, M.W. Long, K.D. Hankenson, Osteogenic differentiation of human mesenchymal stem cells is regulated by bone morphogenetic protein-6, J. Cell. Biochem. 98 (2006) 538.
[9] B. Shen, A. Wei, S. Whittaker, et al., The role of BMP-7 in chondrogenic and osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells in vitro, J. Cell. Biochem. 109 (2010) 406–416.
[10] B. Schmitt, J. Ringe, T. Häupl, et al., BMP2 initiates chondrogenic lineage development of adult human mesenchymal stem cells in high-density culture, Differentiation 71 (2003) 567–577.
[11] E. Alsberg, K. Anderson, A. Albeiruti, et al., Cell-interactive alginate hydrogels for bone tissue engineering, J. Dent. Res. 80 (2001) 2025.
[12] S.R. Chastain, A.K. Kundu, S. Dhar, et al., Adhesion of mesenchymal stem cells to polymer scaffolds occurs via distinct ECM ligands and controls their osteogenic differentiation, J. Biomed Mater. Res. A 78 (2006) 73–85.
[13] R.M. Salasznyk, R.F. Klees, A. Boskey, et al., Activation of FAK is necessary for the osteogenic differentiation of human mesenchymal stem cells on laminin-5, J. Cell. Biochem. 100 (2007) 499–514.
[14] R. McBeath, D.M. Pirone, C.M. Nelson, Et al., Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment, Dev. Cell 6 (2004) 483–495.
[15] A.J. Engler, S. Sen, H.L. Sweeney, et al., Matrix elasticity directs stem cell lineage specification, Cell 126 (2006) 677–689.
[16] A.Y. Thompson, K.A. Piez, S.M. Seyedin, Chondrogenesis in agarose gel culture: a model for chondrogenic induction, proliferation and differentiation, Exp. Cell Res. 157 (1985) 483–494.
[17] A.E. Denker, S.B. Nicoll, R.S. Tuan, Formation of cartilage-like spheroids by micromass cultures of murine C3H10T1/2 cells upon treatment with transforming growth factor-β1, Differentiation 59 (1995) 25–34.
[18] H.L. Ma, S.C. Hung, S.Y. Lin, et al., Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads, J. Biomed Mater. Res. A 64 (2003) 273–281.
[19] H.A. Awad, M. Quinn Wickham, H.A. Leddy, et al., Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds, Biomaterials 25 (2004) 3211–3222.
[20] A.M. DeLise, R.S. Tuan, Alterations in the spatiotemporal expression pattern and function of N-cadherin inhibit cellular condensation and chondrogenesis of limb mesenchymal cells in vitro, J. Cell. Biochem. 87 (2002) 342–359.
[21] J.T. Connelly, A.J. García, M.E. Levenston, Inhibition of in vitro chondrogenesis in RGD-modified three-dimensional alginate gels, Biomaterials 28 (2007) 1071–1083.
[22] U.R. Goessler, P. Bugert, K. Bieback, et al., Integrin expression in stem cells from bone marrow and adipose tissue during chondrogenic differentiation, Int. J. Mol. Med. 21 (2008) 271–279.
[23] R.F. Loeser, Integrins and cell signaling in chondrocytes, Biorheology 39 (2002) 119–124.
[24] P.A. Zuk, M. Zhu, P. Ashjian, et al., Human adipose tissue is a source of multipotent stem cells, Mol. Biol. Cell 13 (2002) 4279.
[25] B.T. Estes, A.W. Wu, F. Guilak, Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6, Arthritis Rheum. 54 (2006) 1222–1232.
[26] P. Malladi, Y. Xu, M. Chiou, et al., Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells, Am. J. Physiol. Cell Physiol. 290 (2006) C1139–C1146.
[27] A.M. Mackay, S.C. Beck, J.M. Murphy, et al., Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow, Tissue Eng. 4 (1998) 415–428.
[28] J.L. Dragoo, J.Y. Choi, J.R. Lieberman, Et al., Bone induction by BMP-2 transduced stem cells derived from human fat, J. Orthop. Res. 21 (2003) 622–629.
[29] B. Peterson, J. Zhang, R. Iglesias, et al., Using genetically modified mesenchymal stem cells from human adipose tissue, Tissue Eng. 11 (2005) 120–129.
[30] D.C. Wan, Y.Y. Shi, R.P. Nacamuli, et al., Osteogenic differentiation of mouse adipose-derived adult stromal cells requires retinoic acid and bone morphogenetic protein receptor type IB signaling, Proc. Natl. Acad. Sci. USA 103 (2006) 12335.

[31] X. Guo, H. Park, G. Liu, et al., In vitro generation of an osteochondral construct using injectable hydrogel composites encapsulating rabbit marrow mesenchymal stem cells, Biomaterials 30 (2009) 2741–2752.
[32] J. Li, S. Mareddy, D.M. Tan, et al., A minimal common osteochondrocytic differentiation medium for the osteogenic and chondrogenic differentiation of bone marrow stromal cells in the construction of osteochondral graft, Tissue Eng. Part A 15 (2009) 2481.
[33] E. Kolettas, H. Muir, J. Barrett, et al., Chondrocyte phenotype and cell survival are regulated by culture conditions and by specific cytokines through the expression of Sox-9 transcription factor, Rheumatology 40 (2001) 1146.
[34] P. Bernard, P. Tang, S. Liu, et al., Dimerization of SOX9 is required for chondrogenesis, but not for sex determination, Hum. Mol. Genet. 12 (2003) 1755.
[35] E. Wright, M.R. Hargrave, J. Christiansen, et al., The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos, Nat. Genet. 9 (1995) 15–20.
[36] B.K. Zehentner, C. Dony, H. Burtscher, The transcription factor Sox9 is involved in BMP-2 signaling, J. Bone Miner. Res. 14 (1999) 1734–1741.
[37] H. Akiyama, J.E. Kim, K. Nakashima, et al., Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors, Proc. Natl. Acad. Sci. USA 102 (2005) 14665.
[38] P. Leucht, S. Minear, D. Ten Berge, et al., Translating insights from development into regenerative medicine: the function of Wnts in bone biology, Semin. Cell Dev. Biol. 19 (2008) 434–443.
[39] G. Zhou, Q. Zheng, F. Engin, et al., Dominance of SOX9 function over RUNX2 during skeletogenesis, Proc. Natl. Acad. Sci. USA 103 (2006) 19004.