

Identification of Bone Marrow-Derived Soluble Factors Regulating Human Mesenchymal Stem Cells for Bone Regeneration

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SUMMARY

Maintaining properties of human bone marrow-derived mesenchymal stem cells (BMSCs) in culture for regenerative applications remains a great challenge. An emerging approach of constructing a culture environment mimicking the bone marrow niche to regulate BMSC activities has been developed. In this study, we have demonstrated a systematic approach to identify soluble factors of interest extracted from human bone marrow and used them in BMSC culture for tissue regeneration. We have found that lipocalin-2 and prolactin are key factors in bone marrow, involved in regulating BMSC activities. Treating the cell with lipocalin-2 and prolactin delays cellular senescence of BMSCs and primes the cell for osteogenesis and chondrogenesis. We have also demonstrated that BMSCs pretreated with lipocalin-2 and prolactin can enhance the repair of calvarial defects in mice. Together, our study provides research evidence of using a viable approach to prime BMSC properties in vitro for improving cell-based tissue regeneration in vivo.

INTRODUCTION

Bone marrow-derived mesenchymal stem cells (BMSCs) are capable of self-renewal and differentiating into multiple cell lineages, including osteoblasts, chondrocytes, and adipocytes (Friedenstein et al., 1987; Johnstone et al., 1998; Pittenger et al., 1999). These features have made BMSCs a promising cell source for clinical applications of cell-based therapies or tissue engineering (Barry and Murphy, 2004; Jorgensen et al., 2004). However, several challenges still remain before the cell can be used satisfactorily for therapeutic applications. The major issue lies in the inevitable procedure of cell expansion in culture after BMSCs are harvested from bone marrow. The cell gradually loses the proliferation and differentiation potential and becomes senescent during the process of cell culture (Palumbo et al., 2014; Wagner et al., 2008).

A stem cell niche is an anatomical location in which stem cells reside and contains physical and chemical components, including soluble factors, extracellular matrix and adhesion molecules, and cells (Schofield, 1978). These components work integratively to maintain quiescence of stem cells to preserve their properties, or to induce migration, proliferation, and differentiation of the cell in response to physiological needs (Jones and Wagers, 2008; Morrison and Spradling, 2008). It is therefore important to study compositions and properties of stem cell niches to obtain deeper insights into how the niche regulates stem cells. Recently, researchers using genetic labeling and cell tracking have demonstrated that the behavior of BMSCs in bone marrow is different from that of BMSCs in culture (Mendez-Ferrer et al., 2010;

Park et al., 2012; Zhou et al., 2014). While how BMSCs are regulated in the bone marrow niche is not fully understood, the findings of these studies suggest that regulation of BMSC activities may be greatly dependent on the interaction between the cell and its niche in bone marrow.

One viable strategy to effectively maintain BMSC properties in culture is to construct a culture microenvironment capable of mimicking the stem cell niche in bone marrow. In addition to the strategies of using hypoxic culture (Palumbo et al., 2014; Tsai et al., 2011) and constructing a physical environment close to the bone marrow niche (Chen et al., 2007; Lai et al., 2010), researchers have used pharmacological approaches to maintain BMSC properties in culture. They treated the cell in culture with the growth factors that have been identified in bone marrow (Gharibi and Hughes, 2012; Handorf and Li, 2011). While these studies have independently shown that a specific soluble factor is able to support proliferation and differentiation of BMSCs, given that bone marrow contains a number of soluble factors, using a single factor in culture is still far off from closely constructing the chemical microenvironment of bone marrow.

In this study, we hypothesized that soluble factors from bone marrow can regulate activities of BMSCs to maintain their properties in culture for multilineage differentiation. To test this hypothesis, we used a systematic strategy by which soluble factors were extracted from bone marrow and analyzed to identify molecules of interest in bone marrow extract. We further evaluated effects of different combinations of the identified molecules on BMSCs to



select a combination that induces the effect closely resembling that of bone marrow extract in regulation of BMSC activities and then used the selected soluble factors to prime the cell for the repair of calvarial defects in mice.

RESULTS

Bone Marrow Extract Maintains Mesenchymal Stem Cell Phenotypes and Primes BMSCs for Osteochondro-Lineage

To determine whether BMSC activities in culture can be regulated by the chemical component of whole bone marrow, we first extracted all of the soluble factors from human bone marrow using PBS with protease inhibitors (Peters et al., 1995; Vituri et al., 2000). Analysis of bone marrow extract by electrophoresis showed that a number of soluble factors were successfully extracted from the bone marrow sample (Figure S1). We next treated BMSCs with bone marrow extract in culture and found that BMSCs treated with 300 or 600 $\mu\text{g}/\text{mL}$ of bone marrow extract showed a more spindle-shaped cell morphology than those without treatment (Figure 1A). However, BMSC numbers in the culture with 600 $\mu\text{g}/\text{mL}$ bone marrow extract were lower than those in the culture with 300 $\mu\text{g}/\text{mL}$ bone marrow extract (Figure 1B), suggesting that the high concentration of bone marrow extract is not as effective as the low concentration in supporting cell proliferation. We thus decided to use the concentration of 300 $\mu\text{g}/\text{mL}$ bone marrow extract, which was approximately 1.5% bone marrow extract in culture medium, for the subsequent experiments.

We then used PCR arrays to analyze the mRNA levels of 84 human mesenchymal stem cell (MSC)-related genes in BMSCs treated with or without bone marrow extract for two cell passages. The mRNA level of each gene in BMSCs treated with bone marrow extract was normalized with that of control BMSCs, and the fold change and p value of each transcript between cells treated with or without bone marrow extract were plotted in a volcano plot (Figure 1C). The treatment of bone marrow extract significantly regulated the expression of 20 transcripts in BMSCs (Figure 1D and Table S1). Specifically, a 4-fold increase in the expression of potency-related *SOX2* was shown in BMSCs treated with bone marrow extract compared with the control cells without treatment of the extract, suggesting that soluble factors in the bone marrow may be able to maintain the stemness of BMSCs (Figure 1D). We also found that bone marrow extract increased the mRNA levels of *CBFA1* and *SOX9* in BMSCs, suggesting that BMSCs may be primed by bone marrow extract for induction of osteogenesis and chondrogenesis (Figure 1D).

Bone Marrow Extract Maintains Properties of BMSCs in Culture

We next asked whether bone marrow extract can maintain properties of BMSCs in culture. To answer this question, we isolated primary BMSCs from bone marrow and selected the $\text{CD271}^+/\text{CD45}^-$ mononuclear cell population using fluorescence-activated cell sorting (FACS) (Figure 1E), since it has been reported that CD271 can be used to identify uncultured BMSCs (Churchman et al., 2012). The transcriptional profile of the BMSC subpopulation was analyzed using the PCR array and then compared with that of cultured BMSCs. Specifically, we performed Spearman's rank correlation analysis to compare the mRNA expression of the 20 genes regulated by bone marrow extract (Figure 1D) in uncultured BMSCs with that of BMSCs cultured with or without bone marrow extract. The Spearman correlation coefficient between the transcriptional profiles of uncultured BMSCs and BMSCs cultured without bone marrow extract was smaller than that between the transcriptional profiles of uncultured BMSCs and BMSCs cultured with bone marrow extract (Figure 1F), suggesting that BMSCs cultured with bone marrow extract maintain the mRNA phenotype of MSC-related genes.

Bone Marrow Extract Increases Proliferation and Delays Cellular Senescence of BMSCs

We further investigated the effects of bone marrow extract on the growth of BMSCs. BMSCs maintained with bone marrow extract increased more rapidly, which resulted in more cumulative population doublings than the cell without bone marrow extract after 100 days of culture (Figures 2A and 2B), suggesting that treatment of bone marrow extract can promote BMSC proliferation and prolong cell growth in culture. To determine the effects of bone marrow-soluble factors on cellular senescence of BMSCs, we also analyzed the activity of senescence-associated β -galactosidase (β -gal) in cells treated with or without bone marrow extract. With treatment of bone marrow extract, fewer cells were stained positive with β -gal than in the culture without bone marrow extract (Figure 2C). The mRNA levels of senescence-related *p16* and *p21* in BMSCs treated with bone marrow extract were lower than those in control BMSCs (Figure 2D), suggesting that bone marrow extract delays cellular senescence of BMSCs in culture.

Pretreatment with Bone Marrow Extract Directs Cell Fate of BMSCs toward Osteogenic and Chondrogenic Lineages

We next evaluated the effects of bone marrow extract on determining the cell fate of BMSCs. The cell was cultured with or without bone marrow extract for two cell passages and then induced without supplementation of

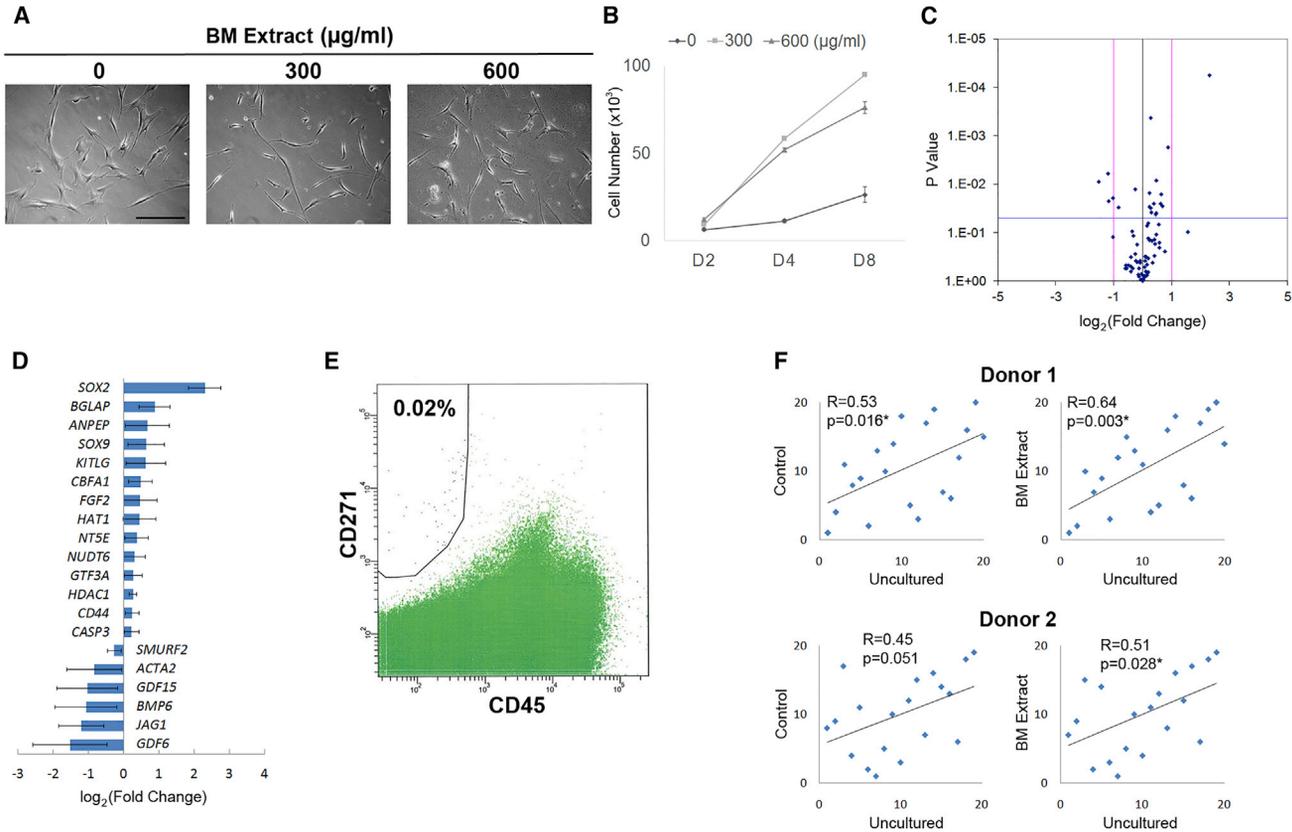


Figure 1. Effects of Bone Marrow Extract on Morphology and Transcript Expression of BMSCs

(A) BMSCs treated with different concentrations of bone marrow extract. Scale bar, 200 μ m.

(B) Numbers of BMSCs cultured with different concentrations of bone marrow extract were quantified. $n = 3$ technical replicates.

(C and D) The mRNA expression of MSC-associated genes in BMSCs from seven donors treated with bone marrow extract detected by a PCR array was normalized to that in control BMSCs. The data were analyzed by paired t test. The average of \log_2 (fold change) or p value in the expression level of mRNA was calculated and plotted in a volcano plot (C). Selected transcripts with a p value of less than 0.05 were plotted in a bar graph (D).

(E) The CD271⁺/CD45⁻ cell population was sorted from bone marrow mononuclear cells using FACS.

(F) Spearman's rank correlation analysis to determine the correlation coefficient between expression levels of selected transcripts in uncultured BMSCs and that in cultured BMSCs with or without bone marrow extract. Each dot representing one transcript in (D) was assigned a number between 1 and 20 corresponding to its position in the ranking based on expression levels of 20 transcripts in cells harvested from two independent donors. The data are presented as means \pm SD. * $p < 0.05$.

bone marrow extract during differentiation. After 21 days of osteogenic induction, the mRNA levels of bone-related markers and the level of calcium deposits in BMSCs pretreated with bone marrow extract were higher than those in control cells (Figures 3A–3C). For adipogenesis, BMSCs pretreated with bone marrow extract expressed lower mRNA levels of adipocyte-related markers and fewer lipid droplets after 21 days of induction than control cells (Figures 3D–3F). For chondrogenesis, the results showed that the mRNA levels of cartilage-related markers and glycosaminoglycan (GAG) production in BMSCs pretreated with bone marrow extract were higher than those of control BMSCs (Figures 3G–3I). Together, our data indicate that priming BMSCs with bone marrow extract

can direct the cell toward the osteogenic and chondrogenic lineages.

Lipocalin-2 and Prolactin in Bone Marrow Extract Regulate Activities of BMSCs

To determine key soluble factors in bone marrow extract that regulate BMSC activities, we analyzed the composition of bone marrow extract using an antibody array. With a 3-fold increase in expression as the threshold, we identified six proteins present in culture medium supplemented with 300 μ g/mL bone marrow extract to be the molecules of interest. These proteins are S100A12, endothelin-1 (ET1), lipocalin-2 (LCN2), prolactin (PRL), matrix metalloproteinase 9 (MMP9), and S100A8/A9 (Figure 4A). Because the S100

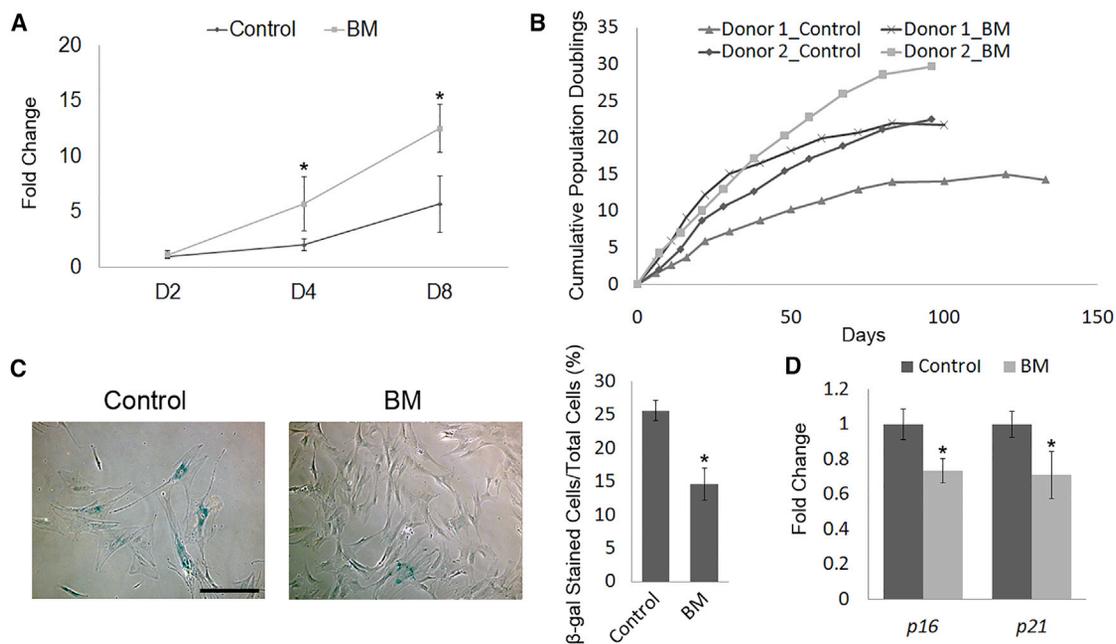


Figure 2. Effects of Bone Marrow Extract on Proliferation and Cellular Senescence of BMSCs

(A) Analysis of cell proliferation of BMSCs cultured with (BM) or without (control) bone marrow extract at days 2, 4, and 8.

(B) Cumulative population doublings of BMSCs cultured with or without bone marrow extract.

(C) Staining of senescence-associated β -gal in BMSCs cultured with or without bone marrow extract (left panels). Scale bar, 200 μ m. β -Gal-positive and total cells were counted and percentage of stained to total cells was calculated (right panel).

(D) qRT-PCR analysis of *p16* and *p21* mRNA levels in BMSCs cultured with or without bone marrow extract.

Paired t test was performed to analyze the data presented as means \pm SD. n = 3 independent donors; *p < 0.05.

family proteins are intracellular proteins and the presence of S100 proteins in bone marrow extract is likely due to cell lysis or necrosis (Riuzzi et al., 2011), we decided to focus on roles of the other four molecules in regulating BMSC activities. The concentration levels of ET1, LCN2, PRL, and MMP9 in the medium supplemented with bone marrow extract were then quantified using ELISA (Table S2).

We next investigated which of the soluble factors in bone marrow extract were involved in regulating BMSC activities. Various combinations of these four selected factors were added to BMSC culture. No increase in cell number was found in the culture treated with any combination of the four selected factors compared with that in the control culture after 5 days (Figure 4B). Based on the results shown in Figure 1D and Table S1, we then selected seven transcripts with a p value less than 0.015, *HDAC1*, *BGLAP*, *CBFA1*, *SOX2*, *GDF6*, *SMURF2*, and *JAG1*, to determine which of the combinations could closely resemble bone marrow extract. Specifically, we quantified the mRNA expression of these seven genes in BMSCs treated with different combinations of the four selected factors and in the cell treated with or without bone marrow extract. Hierarchical clustering showed that the transcriptional profile of BMSCs treated with the combination of LCN2 and PRL

was most similar to that of BMSCs treated with bone marrow extract (Figure 4C, left). The Spearman rank correlation coefficient between BMSCs treated with bone marrow extract and those treated with LCN2 and PRL combined, was higher than that between BMSCs treated with bone marrow extract and those treated with either LCN2 or PRL alone (Figure 4C, right). These results suggest that the combination of LCN2 and PRL most closely resemble bone marrow extract in regulating mRNA expression of the seven selected markers compared with other combinations of the four selected factors.

LCN2 and PRL Delays Cellular Senescence of BMSCs and Directs Cell Fate of BMSCs toward Osteogenic and Chondrogenic Lineages

We then assessed the effect of LCN2 and PRL on cellular senescence of BMSCs. We found that β -gal staining was less and mRNA levels of senescence-related markers were lower in BMSCs cultured with LCN2 and PRL than those in control cells, but more and higher than those in bone marrow extract-treated cells (Figures 5A and 5B). These results suggest that the combination of LCN2 and PRL, similar to bone marrow extract, is able to delay cellular senescence of BMSCs.

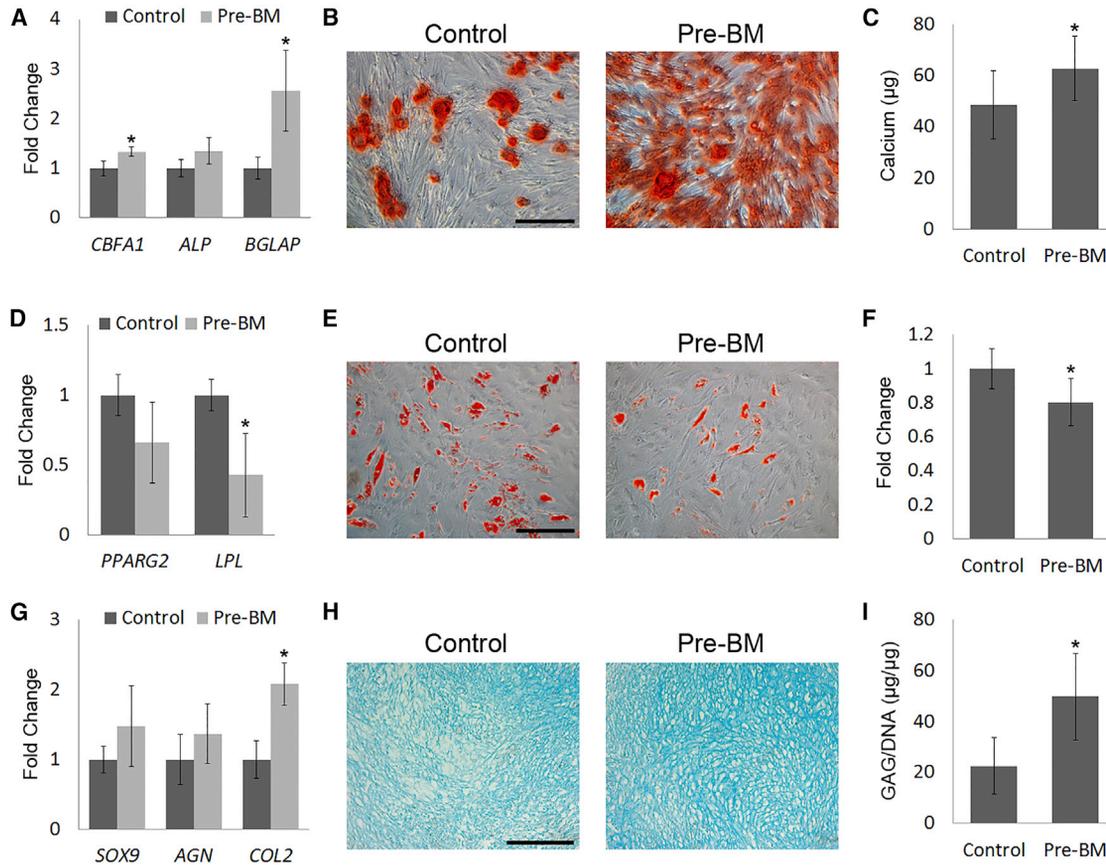


Figure 3. Effects of Bone Marrow Extract Pretreatment on Multilineage Differentiation of BMSCs

BMSCs with (pre-BM) or without (control) bone marrow extract pretreatment for two passages were induced for osteogenesis (A–C), adipogenesis (D–F), or chondrogenesis (G–I) for 21 days.

(A–C) Osteogenesis was evaluated by analyzing relative mRNA expression levels of bone-related markers (A) or Alizarin red staining (B) and quantification of calcium deposits (C).

(D–F) Adipogenesis was evaluated by analyzing relative mRNA levels of fat-related markers (D) or Oil Red O staining (E) and quantification of Oil Red O staining (F).

(G–I) Chondrogenesis was assessed by analyzing relative mRNA expression levels of cartilage-related markers (G) or Alcian blue staining (H) and quantification of GAG production (I).

Paired t test was performed to analyze the data presented as means \pm SD. $n = 3$ independent donors; $*p < 0.05$. Scale bars, 200 μ m.

We further evaluated the effect of LCN2 and PRL on directing tissue-specific fate of BMSCs. After treatment with or without LCN2 and PRL or with bone marrow extract for two cell passages, BMSCs were then induced by differentiation medium without supplementation of LCN2 and PRL or bone marrow extract. After 21 days of osteogenic induction, BMSCs pretreated with LCN2 and PRL showed higher mRNA levels of bone-related markers and calcium deposits than control BMSCs (Figures 5C–5E). In contrast, after 21 days of adipogenic induction, the mRNA levels of adipocyte-related markers and deposition of lipid droplets in BMSCs pretreated with LCN2 and PRL were lower than those in control BMSCs (Figures 5F–5H). For analysis of chondrogenesis, after 21 days of induction, chondrogenic

cell pellets of BMSCs pretreated with LCN2 and PRL showed higher mRNA levels of cartilage-related markers and GAG accumulation than those of control BMSCs (Figures 5I–5K). Together, these results suggest that the treatment of LCN2 and PRL, similar to the treatment of bone marrow extract, can enhance the osteogenic and chondrogenic capacity but reduce the adipogenic capacity of pre-differentiated BMSCs.

Pretreatment of LCN2 and PRL Enhances Bone-Forming Capacity of BMSCs

To investigate the potential of using LCN- and PRL-pretreated BMSCs for repair of bone defects, we performed an in vivo experiment by creating critical-size defects in

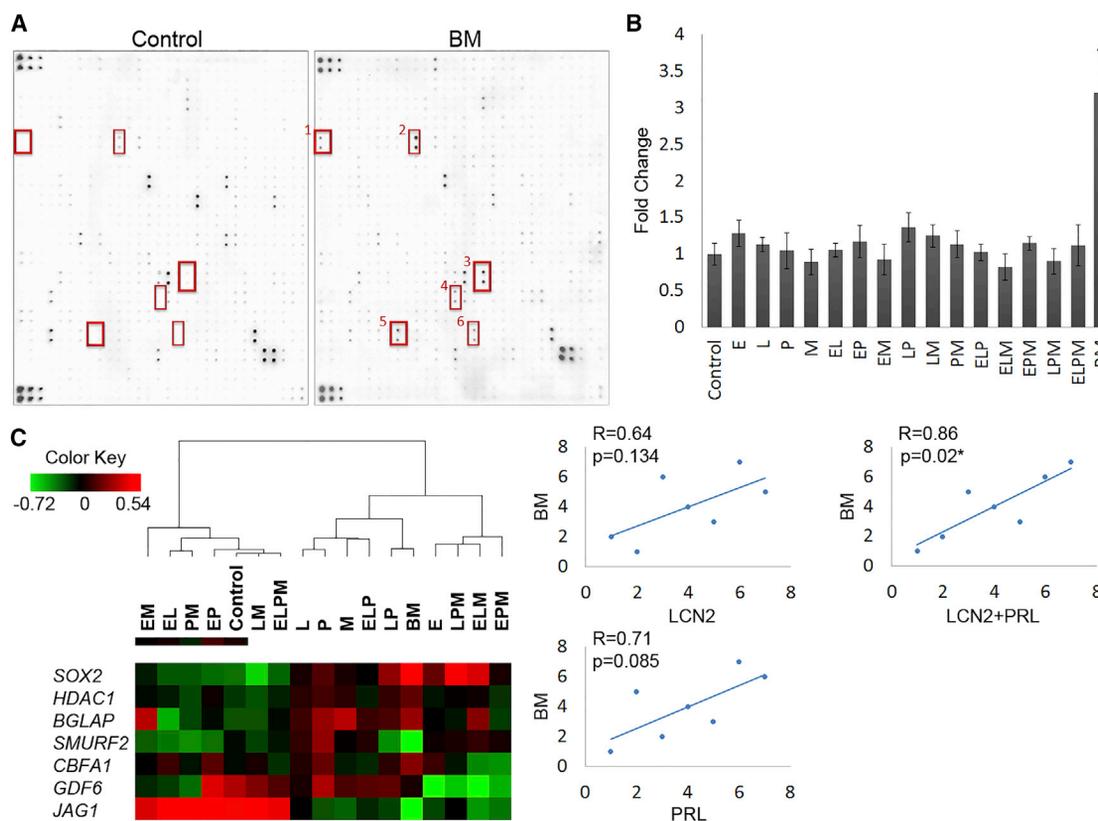


Figure 4. Identification of Soluble Factors of Interest in Bone Marrow Extract

(A) Six proteins in bone marrow extract (BM) expressing at least a 3-fold increase compared with those in control were identified using an antibody array. 1, S100A12; 2, endothelin-1; 3, lipocalin-2; 4, matrix metalloproteinase 9; 5, prolactin; 6, S100A8/A9.

(B) Relative cell numbers in culture treated with various combinations of identified soluble factors for 5 days were quantified. Paired t test was performed to analyze the data presented as means \pm SD. $n = 3$ independent donors; * $p < 0.05$.

(C) Analysis of expression of seven selected transcripts in BMSCs treated with various combinations of identified soluble factors. The dendrogram illustrates a hierarchical clustering calculation of average expression levels of the seven transcripts in the heatmap constructed by assigning different colors based on the calculation by which the expression level of a transcript in one of the groups was centered to the median of expression levels of the transcript in all of the groups. Colors were assigned as follows: black = 0, red > 0 , green < 0 . Spearman's rank correlation was used to determine the correlation coefficient between expression levels of selected transcripts in BMSCs treated with bone marrow extract and those in the cells treated with LCN2, PRL, or LCN2 + PRL (right panels). Each dot representing one transcript in the heatmap was assigned a number between 1 and 7 corresponding to its position in the ranking based on expression levels of seven transcripts in cells harvested from three independent donors. * $p < 0.05$. E, endothelin-1; L, lipocalin-2; P, prolactin; M, matrix metalloproteinase 9.

the calvarial bone of mice and then implanting hydroxyapatite/citric acid-conjugated chitosan (HA/CC) scaffolds seeded with bone marrow extract-treated or LCN2- and PRL-pretreated BMSCs at the defects. HA/CC scaffolds were able to support osteogenesis of BMSCs in vitro, as demonstrated by another independent evaluation (Figure S2). The results of micro-computed tomography (micro-CT) scanning showed a greater radiopaque area in the defect implanted with BMSCs pretreated with LCN2 and PRL than that in the defect implanted with control BMSCs (Figure 6A). Interestingly, the defect implanted with BMSCs pretreated with bone marrow extract showed the

smallest radiopaque mass among the three treatment groups (Figure 6A). The bone volume in the group with BMSCs pretreated with LCN2 and PRL was significantly higher than that in the group with control BMSCs, and the bone volume in the group with BMSCs pretreated with bone marrow extract was the lowest among the three groups (Figure 6B).

We further analyzed the repaired tissue with histological staining. Staining with H&E showed newly formed bone in the repaired tissue with the implant loaded with BMSCs pretreated with LCN2 and PRL. Predominantly cartilage formation without mature bone was found in the repaired

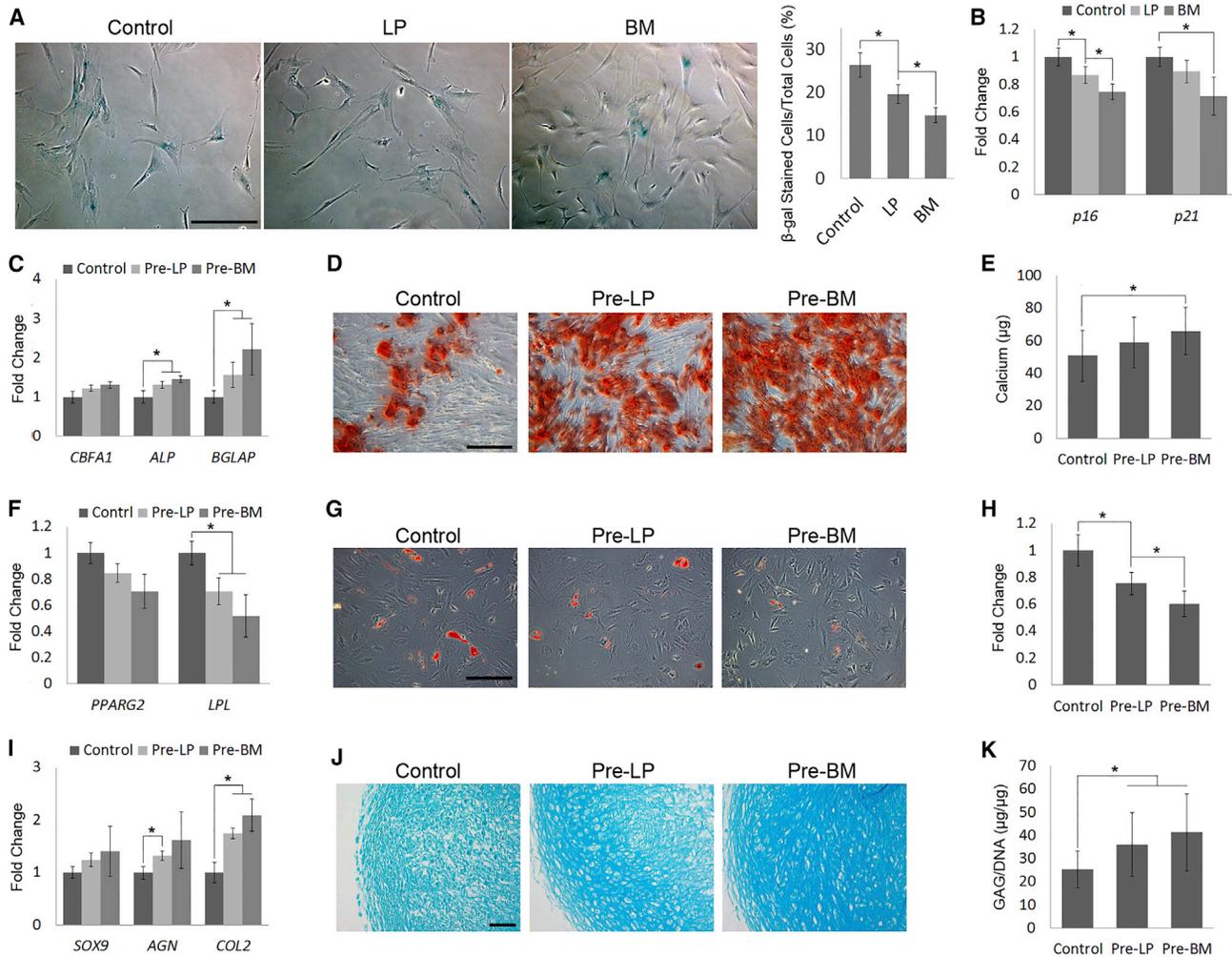


Figure 5. Effects of LCN2 and PRL on Cellular Senescence and Differentiation of BMSCs

(A) Staining of senescence-associated β -gal in BMSCs cultured with LCN2 and PRL or with bone marrow extract (left panels). Scale bar, 200 μ m. Numbers of β -gal-stained and total cells were counted and the ratio was calculated (right panel).

(B) qRT-PCR analysis of *p16* and *p21* mRNA levels in BMSCs cultured with LCN2 and PRL or with bone marrow extract.

(C–K) BMSCs pretreated with LCN2 and PRL (Pre-LP) or with bone marrow extract (Pre-BM) for two passages were induced for osteogenesis (C–E), adipogenesis (F–H), or chondrogenesis (I–K) for 21 days. (C–E) Osteogenesis was evaluated by analyzing relative mRNA expression levels of bone-related markers (C), Alizarin red staining (D) and quantification of calcium deposits (E). (F–H) Adipogenesis was evaluated by analyzing relative mRNA levels of fat-related markers (F), Oil Red O staining (G), and quantification of Oil Red O staining (H). (I–K) Chondrogenesis was assessed by analyzing relative mRNA expression levels of cartilage-related markers (I), Alcian blue staining (J), and quantification of GAG production (K).

Two-way ANOVA without replication with post hoc Tukey's test was performed to analyze the data presented as means \pm SD. $n = 3$ independent donors; * $p < 0.05$. Scale bars, 200 μ m.

tissue with the implant loaded with control BMSCs or BMSCs pretreated with bone marrow extract (Figure 6C). We also analyzed the expression of bone-associated osteopontin (OPN) and osteocalcin (OC) using immunohistochemistry. Interestingly, the newly formed bone at defects repaired by the implant loaded with BMSCs pretreated with LCN2 and PRL was not stained positive for human-specific OPN or OC (Figure S3), indicating that the formation of

new bone resulted from activities of host MSCs that were possibly recruited by exogenous BMSCs pretreated with LCN2 and PRL. Nevertheless, the staining of OPN and OC was found in the cartilage matrix of repaired tissue, demonstrating that the cartilage tissue was undergoing ossification (Figure 6D). The staining intensities of OPN and OC in sections of the tissue repaired by BMSCs pretreated with LCN2 and PRL were greater than those in

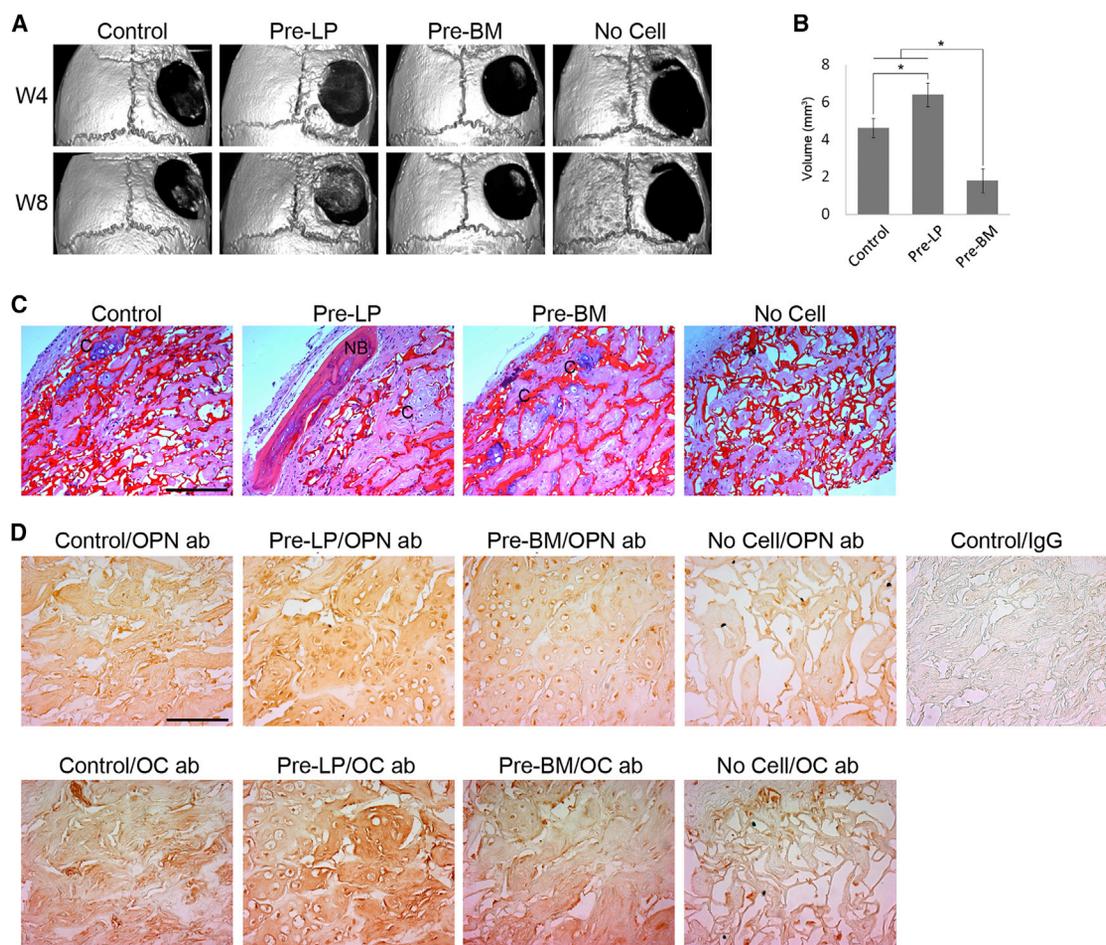


Figure 6. Micro-CT and Histological Analysis of Bone Regeneration in Mouse Calvarial Defects

(A) Micro-CT images of calvarial defects taken 4 and 8 weeks after implantation of BMSC-seeded HA/CC scaffolds or an acellular scaffold (No Cell). Cells were pretreated with LCN2 and PRL (Pre-LP), with bone marrow extract (Pre-BM), or without either of these two (Control). (B) Quantification of volume of newly formed bone in a defect 8 weeks post implantation. One-way ANOVA with post hoc Tukey's test was performed to analyze the data from four mice presented as means \pm SD. $n = 4$; $*p < 0.05$. (C) H&E staining of calvarial defects repaired by BMSC-seeded HA/CC scaffolds for 8 weeks. NB, newly formed bone (red color); C, cartilage (purple color). Scale bar, 200 μ m. (D) Immunohistochemical localization of OPN and OC in calvarial defects repaired by BMSC-seeded HA/CC scaffolds for 8 weeks. ab, antibody; IgG, immunoglobulin G. Scale bar, 100 μ m.

sections of the tissue repaired by control or bone marrow extract-pretreated BMSCs (Figure 6D), suggesting that LCN2 and PRL in culture can enhance the bone-forming capability of cultured BMSCs, which in turn increases bone formation during tissue repair.

Pretreatment with Bone Marrow Extract Increases the Migratory Capacity of BMSCs

As described earlier, our results showed an interesting finding that scaffolds with BMSCs pretreated with bone marrow extract were less supportive of new bone formation during tissue repair than the other two groups. Based on this finding, we hypothesized that pretreatment with

bone marrow extract enhances the migratory capacity of BMSCs, thus promoting BMSCs to migrate out from a scaffold in vivo, resulting in reduced bone formation. We tested the mobility of BMSCs pretreated with or without LCN2 and PRL or with bone marrow extract using the transwell migration assay. The number of migrated BMSCs pretreated with bone marrow extract was significantly higher than that of migrated control BMSCs or that of migrated BMSCs pretreated with LCN2 and PRL (Figures 7A and 7B). The mRNA level of *CXCR4*, a key molecule involved in cell migration (Cheng et al., 2008; Shi et al., 2007), in BMSCs treated with bone marrow extract was also significantly upregulated (Figure 7C). These results

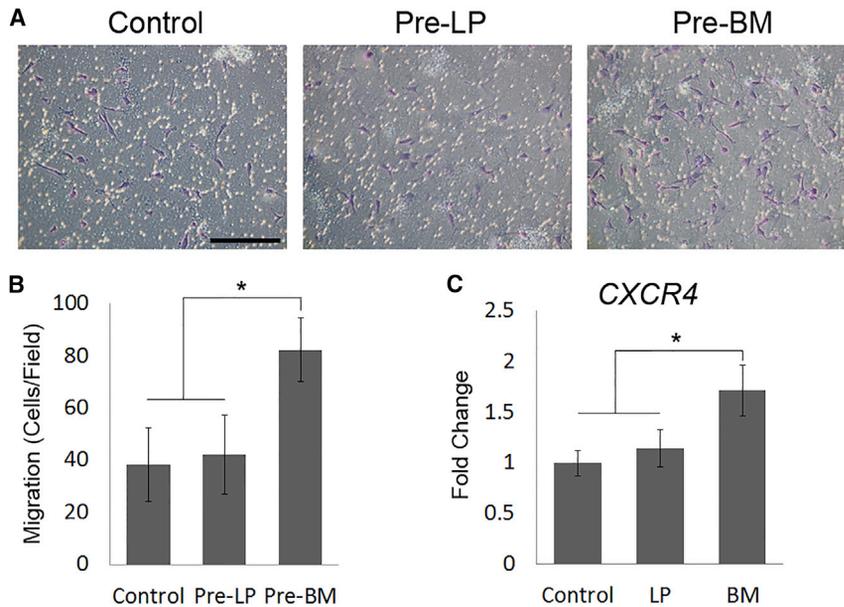


Figure 7. Migration of BMSCs in Transwell Plates

(A) Crystal violet staining of transmigrated BMSCs. Scale bar, 200 μ m.

(B) Transmigrated BMSCs were quantified by counting cells in four randomly selected areas of the visual field under a microscope. (C) Relative expression levels of *CXCR4* in BMSCs treated with or without LCN2 and PRL or with bone marrow extract.

Two-way ANOVA without replication with post hoc Tukey's test was performed to analyze the data presented as means \pm SD. $n = 3$ independent experiments; * $p < 0.05$.

provide a possible explanation of why less bone was formed in calvarial defects repaired by scaffolds seeded with BMSCs pretreated with bone marrow extract than that in defects repaired by scaffolds with control BMSCs or BMSCs pretreated with LCN2 and PRL.

DISCUSSION

Constructing a culture environment outside the body to imitate a stem cell niche is an emerging strategy to study stem cell behavior in a controllable manner (Brafman, 2013; Ranga et al., 2014; Torisawa et al., 2014). In this study, we have developed an approach to construct a chemically defined culture environment for BMSCs through a systematic methodology to determine the effects of bone marrow extract on BMSCs in culture, identify molecules involved in the regulation, and evaluate the potential of using the identified molecules to prime BMSCs for tissue repair. Our results show that the cell culture supplemented with LCN2 and PRL can at least partially mimic the chemical microenvironment of bone marrow, and human BMSCs maintained in our chemically defined culture are capable of enhancing bone formation in a critical-size defect.

In this study, we compare the transcriptional profile of BMSCs treated with or without bone marrow extract with that of uncultured $CD271^+/CD45^-$ BMSCs to demonstrate that cultured BMSCs treated with bone marrow extract may be able to maintain the property of naive human BMSCs. Specifically, we have found that induced by bone marrow extract, the expression level of potency-related *SOX2* in

cultured BMSCs is increased to maintain the stemness property. However, it is unclear why cell lineage-associated transcription factors *CBFA1* and *SOX9* are also upregulated in response to induction of bone marrow extract. This particular finding is similar to that of a previous report (Churchman et al., 2012). One possible explanation may be because different cell subpopulations in heterogeneous BMSCs are induced by bone marrow extract to upregulate simultaneous expression of the stemness and lineage-associated genes. On the other hand, we have found that the expression levels of *BMP6*, *GDF6*, and *GDF15* were decreased in BMSCs treated with bone marrow extract. *BMP6* is a potent inducer for osteogenesis of BMSCs (Sammons et al., 2004). *GDF15* can also induce BMSCs toward osteogenic lineage (Uchiyama et al., 2015), and *GDF6* is involved in adipogenesis and chondrogenesis of BMSCs (Wang et al., 2013; Clarke et al., 2014). Our finding that bone marrow extract downregulates the expression of *BMP6*, *GDF6*, and *GDF15* in BMSCs suggests that reduction of these regulatory molecules may be critical in maintaining BMSCs with undifferentiated status.

One of the key findings of this study is that LCN2 and PRL play critical roles in regulation of BMSC activities. LCN2 in bone marrow is secreted by adipocytes (Yan et al., 2007; Zhang et al., 2008) and neutrophils (Kjeldsen et al., 1994), and can function as an inflammatory cytokine of the innate immune system to limit bacterial growth by sequestering iron-containing siderophores (Yang et al., 2002). It can also form a complex with MMP9 to protect the activity of MMP9, which causes loss of cartilage matrix in osteoarthritis (Gupta et al., 2007). Halabian et al. (2013) have demonstrated that LCN2 can upregulate antioxidant



activities and increase production of growth factors in BMSCs. Another study by Bahmani et al. (2014) has shown that LCN2 can protect BMSCs from oxidative stress and delay cellular senescence of BMSCs in culture. Recently, Lu et al. (2015) have found that LCN2 can regulate the fate of BMSCs. Treatment with LCN2 primes BMSCs for osteogenesis by upregulating the expression of *CBFA1*. The findings of these reports are in agreement with ours that BMSCs cultured with LCN2-supplemented medium can delay cellular senescence and increase their osteogenic capacity. In addition, our results also show that PRL plays a determinative role in regulating BMSC activities in culture. Prolactin is a hormone primarily released by the pituitary gland to stimulate mammary glands to produce milk (Friesen et al., 1970). Our findings provide evidence that other than autocrine or paracrine molecules, endocrine molecules such as prolactin from a remote tissue source also participate in regulating BMSC activities in the bone marrow niche. As an endocrine molecule, the level of PRL in blood varies depending on the physiological or pathological conditions of an individual. In fact, Wildburger et al. (1998) have reported that an elevated level of PRL in blood is associated with increased osteogenesis in a patient with traumatic injury. An increased level of PRL in plasma of breastfeeding women is also able to upregulate osteogenesis to protect them from developing osteoporosis (Bjornerem et al., 2011; Sowers et al., 1995). With regard to chondrogenesis, a previous study by Ogueta et al. (2002) has shown that PRL can induce BMSCs to increase GAG production during chondrogenic induction. In this study, we have demonstrated that LCN2 and PRL can direct BMSCs toward osteogenic and chondrogenic lineage differentiation, consistent with the previous findings reported by other groups.

Another interesting finding of this study is that bone formation in calvarial defects implanted with scaffolds laden with BMSCs pretreated with bone marrow extract is noticeably less than that in defects implanted with scaffolds laden with BMSCs pretreated with or without LCN2 and PRL. However, the *in vitro* results of osteogenesis in culture have shown that bone marrow extract-pretreated BMSCs produce significantly more calcium deposits in scaffolds than the cells pretreated with or without LCN2 and PRL. The discrepancy in the results may be due to differences in BMSC behavior between the *in vitro* and *in vivo* micro-environments, given that BMSCs are exposed to a number of cytokines and inflammatory molecules in the calvarial defect. We also hypothesized that BMSCs pretreated with bone marrow extract are more migratory and increasingly promoted to migrate out of an implanted scaffold, thus resulting in decreased bone formation in a calvarial defect relative to the cell pretreated with or without LCN2 and PRL. Our cell migration assay with transwell culture has

confirmed this hypothesis. We have also found that the treatment of bone marrow extract can significantly increase the mRNA expression of *CXCR4* in BMSCs to upregulate cell migration, as activation of the *CXCR4/CXCL12* pathway plays a key role in mediating the homing of BMSCs (Cheng et al., 2008; Shi et al., 2007). It is unclear which percentage of bone marrow extract-treated BMSCs in our implanted scaffold is involved in cell migration and where the migrated cells end up in mice. While it would be of interest to investigate migration of bone marrow extract-treated BMSCs after implantation in future studies, our results have undoubtedly demonstrated that LCN2- and PRL-treated BMSCs can enhance repair of a critical-size bone defect without causing the concern of low cell retention within an implanted scaffold.

A limitation of the current study is that bone marrow used in this study is harvested from femoral heads of donors with osteoarthritis. It is unclear how the disease may affect bone marrow compositions and properties and whether bone marrow from an osteoarthritic donor is different from that from a non-osteoarthritic donor. Future studies to confirm our findings using non-osteoarthritic donors' cells may be helpful. Nonetheless, harvesting bone marrow from osteoarthritic donors has been used by several groups, including ours (Narcisi et al., 2015; Tsai et al., 2015) to obtain the tissue for BMSC isolation.

EXPERIMENTAL PROCEDURES

Preparation of Bone Marrow Extract

Human bone marrow tissue was harvested from the inner compartments of femoral heads and necks from seven patients who underwent total hip arthroplasty. The procurement of bone marrow tissue was approved by the UW-Madison Institutional Review Board. Bone marrow extract was prepared following previously published protocols with modifications (Peters et al., 1995; Vituri et al., 2000). In brief, bone marrow was harvested from the interior compartment of femoral necks and heads and mixed with the same volume of PBS containing protease inhibitor cocktail (Roche). After incubation with ice for 30 min, the bone marrow/PBS solution was centrifuged at $2,500 \times g$ for 15 min. The supernatant was collected, filtered through a 0.22- μm membrane, and stored at -20°C for later use. Bone marrow extract of any three of the seven donors was combined and used in this study to eliminate donor variability. The concentration of total protein in bone marrow extract was determined by the Bicinchoninic Acid Assay kit (Pierce).

Isolation of BMSCs from Bone Marrow and Cell Culture

Human BMSCs were isolated from femoral heads of the seven patients following a previously published protocol (Tsai et al., 2015). In brief, harvested bone marrow was thoroughly mixed with 30 mL of DMEM (Gibco). A syringe with an 18-gauge needle



was used to filter out bone debris. Filtered bone marrow was then centrifuged at 1,300 rpm for 5 min to collect cell pellets. After supernatant was removed, a cell pellet was reconstituted in Hank's balanced salt solution (Invitrogen), gently added into a conical tube containing Ficoll gradient solution (GE Healthcare), and centrifuged at $600 \times g$ for 30 min. Mononuclear cells were collected, plated in cell culture flasks (Falcon) with culture medium composed of low-glucose DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco) and antibiotics, and maintained at 37°C in a humidified, 5% CO₂ atmosphere. Cells were trypsinized using 0.05% trypsin/EDTA (Gibco) after reaching 70%–80% density confluence and replated at a seeding density of 1,000 cells/cm². Culture medium was replaced every 3 days.

To study the effects of bone marrow extract on regulation of BMSC activities, we treated BMSCs in culture with or without 300 µg/mL of bone marrow extract for various lengths of time in different experiments. The information of culture time is specified in the section of individual assays.

Fluorescence-Activated Cell Sorting for BMSCs

To harvest naive BMSCs, we isolated mononuclear cells that express the surface antigen CD271 but not CD45 from bone marrow (Churchman et al., 2012). In brief, after bone marrow-derived mononuclear cells were collected using the method described above, the cell was labeled with APC-conjugated anti-CD271 (catalog #560326, BD Biosciences) and PE-conjugated anti-CD45 (catalog #555483, BD Biosciences) antibodies in FACS buffer composed of PBS, 1% BSA, and 5 mM EDTA. The fluorescence minus one control was used to set the gates. After washing with FACS buffer three times to remove unbound antibodies and analysis by a cell sorter (BD Biosciences), approximately 10,000 of the CD271⁺/CD45⁻ cells were recovered. Total RNA was extracted directly from the uncultured CD271⁺/CD45⁻ cells, and transcriptional profiles of these cells were analyzed using human MSC PCR arrays.

Identification of Soluble Factors

To identify soluble factors in bone marrow extract, we used an antibody array capable of detecting 507 human proteins, including growth factors and cytokines, to analyze samples following the manufacturer's instructions (RayBiotech) (Palumbo and Li, 2013). In brief, 10% FBS-supplemented culture medium containing 300 µg/mL bone marrow extract was biotin-labeled and then incubated in an array precoated with target antibodies. Basal culture medium with 10% FBS served as a control. After washing with PBS three times to remove unbound molecules, arrays were incubated with streptavidin conjugated with horseradish peroxidase, visualized with chemiluminescent reagents, and scanned by an imaging system. The signal intensity of each target protein was quantified by the densitometry with ImageJ. Protein levels in culture medium containing bone marrow extract were compared with those in basal culture medium without bone marrow extract to identify proteins of interest that showed at least a 3-fold increase in expression with induction of bone marrow extract. An ELISA was used to confirm the concentration of proteins of interest in bone marrow extract following the manufacturer's instructions.

Creation of Calvarial Defects and Implantation of BMSC-Seeded Scaffolds

All animal studies were performed in accordance with federal regulations and with the approval of the Institutional Animal Care and Use Committee. Male non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (NOD.CB17-Prkdc^{scid}/J) (6–8 weeks old) were purchased from The Jackson Laboratory. A 4-mm-diameter calvarial defect was created unilaterally at the mid-parietal location of a mouse using a round dental burr. The surgery was performed while the animal was anesthetized by isoflurane. Each defect was then filled with a round BMSC-seeded HA/CC disc with dimensions of 4 mm diameter and 1 mm thickness. The cells were maintained with or without 300 µg/mL bone marrow extract or with 18 ng/mL LCN2 and 1 ng/mL PRL for three passages before seeding in HA/CC discs. The cellular scaffolds were maintained in 10% FBS-supplemented culture medium without osteogenic induction for 2 days before implantation. Animals were randomly assigned to four groups: (1) implantation of scaffolds seeded with control BMSCs (n = 4), (2) implantation of scaffolds seeded with BMSCs pretreated with LCN2 and PRL (n = 4), (3) implantation of scaffolds seeded with BMSCs pretreated with bone marrow extract (n = 4), and (4) implantation of a scaffold without BMSCs (n = 1). After surgery, the mice were housed under controlled temperature of 25°C and fed a standard diet for 8 weeks before being euthanized with CO₂.

Histological Analysis of Calvarial Defects Repaired by Implants

Tissue specimens at calvarial defects repaired by HA/CC implants were harvested after animals were euthanized, then fixed with 4% formaldehyde for 24 hr. After decalcification with 5% formic acid for 24 hr, specimens were dehydrated, infiltrated with xylene, embedded in paraffin, and cut into 10-µm sections using a microtome. For histology analysis, sample sections were deparaffinized, rehydrated, and stained with H&E. For immunohistochemistry analysis, rehydrated sections were incubated with 1 µg/mL pepsin (Sigma-Aldrich) in 10 mM hydrochloride for 10 min to retrieve antigens, treated with 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity, and blocked with PBS containing 10% BSA for 30 min. After washing with Tris-buffered saline, the sections were incubated with primary antibody against OPN or OC (Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase-conjugated secondary antibody. The slides were then developed by being incubated with 3,3'-diaminobenzidine substrate solution, dehydrated, mounted, and visualized using a microscope (Nikon, Japan).

Cell Migration Assay

A cell migration assay was performed in a 12-well transwell plate containing track-etched polyethylene terephthalate membranes with 8-µm pores (Falcon). The amount of 20,000 BMSCs pretreated with or without LCN2 and PRL, or with bone marrow extract, was seeded in the upper chamber of a transwell assembly with 200 µL of culture medium. The cells did not undergo serum starvation prior to seeding. The lower chamber contained 1 mL of culture medium. After incubation at 37°C for 3 hr, transwell inserts were removed from the plate and the upper surface of the membrane was scraped



gently with a cotton-tipped applicator to remove the medium and remaining cells. The transwell insert was then treated with 4% formaldehyde for 15 min and stained with 0.5% crystal violet (Sigma-Aldrich) for 10 min. The number of migrated cells was determined by counting cells in four randomly selected areas per well under a microscope.

Statistical Analysis

All quantitative data of assays are presented as the mean \pm SD as the assays were performed with samples in triplicate ($n = 3$), quadruplicate ($n = 4$), or septuplicate ($n = 7$). The paired *t* test or a two-way ANOVA without replication with post hoc Tukey's test was used to analyze quantitative data. For correlation analysis, Spearman's rank correlation was used. A power analysis with the setting of correlation coefficient at 0.8 and *p* value at 0.05 was performed to determine the required number of transcripts that should be selected for determining effects of different combinations of ET1, LCN2, PRL, and MMP9 on BMSCs. A *p* value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2017.01.004>.

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