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Acute Skeletal Injury is Necessary for Human Adipose-Derived Stromal Cell Mediated Calvarial Regeneration

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Abstract

Background—Studies have demonstrated that human adipose derived stromal cells (hASCs) are able to repair acute calvarial injuries. However, the more clinically relevant repair of an established skeletal defect has not been addressed. We sought to determine whether hASCs could heal chronic (established) calvarial defects.

Methods—Critical-sized (4mm) mouse parietal defects were created. hASCs were either engrafted immediately postoperatively (acute defect), or 8 weeks following defect creation (established defect). Methods of analysis included microCT scans, histology, and *in situ* hybridization. Finally, hASCs were treated *in vitro* with PRP to simulate an acute wound environment; proliferation and osteogenic differentiation were assessed (Alkaline phosphatase, Alizarin red, and qRT-PCR).

Results—Near complete osseous healing was observed when calvarial defects were immediately engrafted with hASCs. In contrast, when hASCs were engrafted into established defects, little bone formation occurred. Histological analysis affirmed findings by microCT, showing more robust staining for alkaline phosphatase and picrosirius red in an acute than in a established hASC engrafted defect. *In situ* hybridization and qRT PCR showed an increase in BMP expression (*Bmp2*, *Bmp4* and *Bmp7*) acutely following calvarial defect creation. Finally, *in vitro* treatment of hASCs with PRP enhanced osteogenic differentiation and increased *Bmp2* expression.

Conclusions—While hASCs can be utilized to heal an acute mouse calvarial defect, hASCs do not enhance healing of an established (or chronic) defect. Endogenous BMP signaling activated

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post-injury may explain these differences in healing. Platelet rich plasma enhances osteogenic differentiation of hASCs *in vitro* and may prove a promising therapy for future skeletal tissue engineering efforts.

Keywords

Osteogenesis; Adipose derived mesenchymal cells; Multipotent stromal cells; Platelet rich plasma, Bone morphogenetic protein, Adult Stem Cells

Introduction

The societal burden of diseases afflicting the musculoskeletal system is significant. In 2007, it was estimated that diseases of the musculoskeletal system cost over 26 billion dollars with an average annual growth rate of 8.5%.(1) The practicing plastic surgeon plays a significant role in the repair and/or reconstruction of skeletal defects, whether from acquired or congenital causes. While some bone reconstructions are performed acutely, many patients are seen for reconstruction months to years after the original cancer extirpation or traumatic event. Thus, the study of skeletal tissue regeneration should include not only the treatment of acute bone injuries, but also established or long-standing bone injuries.

Current methods to treat calvarial defects include alloplastic materials and bone grafts which are plagued by rejection and resorption, respectively. Limited clinical trials report an even greater incidence of complications for alloplastic options, leaving surgeons with no optimal treatment alternatives. Therefore, surgeons have begun to pursue other treatment modalities, such as cell-based tissue engineering techniques, to address the shortcomings of the treatment options currently offered.(2) Human adipose-derived stromal cells (hASCs) represent a multipotent stromal cell type with a proven capacity to differentiate along an osteogenic lineage. Despite accumulating data suggesting the potential clinical utility of ASCs in skeletal tissue regeneration, the more clinically relevant repair of a long-standing skeletal defect has, to date, not been addressed in detail.

A critical-sized calvarial defect in the mouse is a reproducible, frequently utilized model for the study of cell-based skeletal repair. In our laboratory, we have demonstrated that ASCs, from mouse or human origin, heal critical size mouse cranial defects. A critical sized (4mm, parietal bone) mouse calvarial defect shows no healing without ASC engraftment up to 16 weeks post injury.(3) However, if hASCs are seeded onto an osteoinductive scaffold, significant bony healing is observed in as little as 4 weeks post injury.(4) ASC mediated calvarial healing has only been observed in an acute injury. In the following study, we sought to examine the differences in healing between a newly created defect and an established (eight week old) calvarial defect using hASCs. In addition, we examined potential underlying mechanisms that may explain the differences we found in ASC mediated healing between an acute and an established osseous defect.

Methods

Chemicals, supplies and animals

Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from GIBCO Life Technologies, (Carlsbad, CA). Platelet rich plasma was obtained from Zen Biosciences. CD-1 wild-type mice, CD-1 nude mice (CrI:CD-1 Foxn1^{nu}), were obtained from Charles Rivers, (Wilmington, MA).

Cell Harvest

Human ASCs were harvested from lipoaspirate derived from four women under 60 years of age, under BMI of 30 kg/m², and from the flank and thigh regions by suction assisted liposuction as previously described (4–6). Briefly, adipose specimens were washed with an equal volume of dilute Betadine, and two phosphate buffered saline (PBS) washes of equal volume to the lipoaspiration specimen. Tissues were subsequently digested with an equal volume of 0.075% (w/v) Type II collagenase in Hank's Balanced Salt Solution at 37.0°C in water bath with agitation at 140 rpm for 60 minutes. Next, the collagenase digest was inactivated with an equal volume of PBS with 10% Fetal Bovine Serum and 1% Pen/Strep. The stromal vascular fraction was then pelleted via centrifugation at 1000 rpm for 6 minutes at 4.0°C. The supernatant was discarded, and the cell pellet re-suspended and filtered through a 100 micrometer cell strainer. The cells were again pelleted and re-suspended in growth media, and primary culture was established in tissue culture plates incubated at 37 °C in an atmosphere of 5% CO₂. Cells were expanded to passage 1 and 2 and subsequently used for *in vitro* and *in vivo* assays.

Preparation of scaffolds

Apatite-coated PLGA scaffolds were fabricated from 85/15 poly(lactic-co-glycolic acid) by solvent casting and a particulate leaching process as previously described(4,7).

For apatite coating, simulated body fluid (SBF) solution was prepared by sequentially dissolving CaCl₂, MgCl₂·6H₂O, NaHCO₃, and K₂HPO₄·3H₂O in ddH₂O as previously described.

Creation and treatment of calvarial defects

Non-healing, critical-sized (4mm) calvarial defects were created in the right parietal bone of adult (60 day-old) male CD-1 nude mice, leaving dura mater undisturbed. Those defects considered acute had a scaffold placed immediately at the time of injury. Osseous defects considered established were left empty having only their skin incision closed following bone defect creation. These mice considered established were followed for 8 weeks by MicroCT scans to ensure that no internal healing had taken place. After eight weeks, the incision was re-opened and debridement of the defect was performed down to the dura mater, the osseous defect still measuring 4mm.

In preparation for cell engraftment, scaffolds were seeded as previously described.(4) Briefly, 150,000 cells are resuspended in 25ul of media and seeded on the PLGA scaffold for 30 minutes. Subsequently, 100ul of standard growth media or standard growth media supplemented with 5% activated PRP for 12 hours. Animals were divided equally into six treatment groups: 1) empty defects in which a 4mm defect was created but left empty (*n*=5), 2) scaffold only, in which a PLGA scaffold without cells was placed in the acute defect site (*n*=5), 3) scaffold only, in which a PLGA scaffold without cells was placed in the established defect site (*n*=5), 4) 150,000 hASCs on a scaffold, in which hASCs were impregnated in a scaffold and then placed in the acute defect site (*n*=5) 5) 150,000 hASCs on a scaffold, in which hASCs were impregnated in a scaffold and then placed in an established defect site (*n*=5) and 6) 150,000 hASCs on a scaffold in which the hASCs were primed with 5% PRP overnight and then placed in an established defect site that was redebrided 1mm (*n*=4).

In vivo imaging

Micro-CT was performed on live animals in a serial manner postoperatively (through 8 weeks healing), using a high-resolution MicroCAT II™ (ImTek Inc., Knoxville, TN) and reconstructed as previously described.(4) Percent healing was determined by dividing the

size of the defect at each time point by the size of the defect at time 0. For the established defect group, time 0 was at 8 weeks prior to scaffold implantation.

Histologic analyses

Up to 16 weeks postoperatively, animals were sacrificed for histology. Calvaria were harvested, formalin-fixed, decalcified in 19% EDTA, paraffin-embedded and sectioned at 8 μ m thickness. Aniline blue staining was performed as previously described (approximately $n=30$ slides per surgical group).(8) Next, select slides were stained with Pentachrome, Alkaline phosphatase and Picrosirius Red.(8,9) Alkaline phosphatase expression was quantified on select slides utilizing the magic wand tool in Adobe Photoshop ($n=5$ slides per group). *In situ* hybridization was performed on select slides for mouse *Bmp2*, *Bmp4* and *Bmp7* as previously described.(8) Non-specific binding was minimized by high stringency hybridization conditions, for all assays sense probes were used side-by-side with minimal background.

Fish Analysis for sex chromosomes

Tissue sections were pretreated by standard protocol using the VP2000™ slide pretreatment instrument (Abbott Molecular) as previously described.(4)

In vitro Assays and Assessments

In order to mimic one aspect of an acute wound environment, the effects of activated human PRP on hASCs were assessed in a series of cell culture assays. First, PRP was activated by the addition of 14.2 U/mL thrombin and 10% CaCl_2 , followed by incubation at 37°C for 30 minutes. PRP was then centrifuged at 1000rpm for 10 minutes and supernatant was used immediately for all experiments.

To assess proliferation, BrdU incorporation and cell counting assays were performed as previously described (10). Medium was replenished every other day, with or without PRP. For cell counting assays, hASCs were seeded 5,000 cells / well in 12-well plates as previously described.(11) Growth medium was replenished every other day with or without PRP.

Cells were seeded onto 6-well plates, 100,000 cells / well, and treated with standard osteogenic differentiation media (ODM) with or without PRP (6) ($N=4$ patients). Medium was changed every 3 days. Alkaline phosphatase staining and quantification was performed at 3 days (5) ($N=4$ patients). Alizarin red staining and photometric quantification was performed at 7 days to assay bone nodule formation (5) ($N=4$ patients). Quantification was performed by cetylpyridinium chloride extraction.

Ribonucleic Acid (RNA) Isolation and Polymerase Chain Reaction

RNA isolation and reverse transcription was performed as previously described (11–13). All PCR products were run out on a 2% agarose gel to determine appropriate size and specificity. Sequences are shown in Supplemental Digital Content 1 - Table 1. Levels of expression were determined by normalizing to *GAPDH*; reactions were performed in triplicate.

Statistical Analysis

Means and standard deviations were calculated from numerical data, as presented in the text, figures and figure legends. In figures, bar graphs represent means, whereas error bars represent one standard deviation. Statistical analysis was performed using an appropriate ANOVA test, as described in the Figure Legends. For data that follow the Poisson

distribution, a square root transformation was employed. In addition, a post-hoc two-tailed Student's *t*-test was used. * $p \leq 0.05$ was considered to be significant.

Results

ASC healing of an acute and established calvarial defects by computed tomography

First, successful hASC *in vivo* cell engraftment was confirmed. PLGA scaffolds were seeded as described in the methods section. Representative animals from each group were sacrificed at 1 week. Fluorescent *in situ* hybridization (FISH) was performed specific for human sex chromosomes. This was performed to confirm viability of directly engrafted female hASCs. Results showed, as expected, that those cells within the defect site were positive for human-X chromosome (see Supplemental Digital Content 2 - Figure 1 shows Fluorescent *in situ* hybridization for human X chromosome, appearing green. Nuclear counterstain appearing blue. As expected, the majority of cells within the defect site at one week were of human origin, showing two X chromosomes. Specificity of FISH analysis was ensured, as sites other than the defect were negative.

We next set out to compare calvarial defect healing upon placement of a hASC seeded osteoconductive scaffold in an acute as compared to a chronic critical sized defect (Fig. 1). In as little as two weeks, hASCs showed significant bone formation within the acute defect site (middle row, Fig. 1A). By four weeks and thereafter, the majority of acute defects treated with hASCs showed robust healing. This was in comparison to established (or 8 week old) hASC engrafted defects which showed little ossification even up to eight weeks, (bottom row, Fig. 1A). These findings were in comparison to defects left empty or defects treated with a scaffold alone: both groups showed significantly less healing (top row and data not shown). Results were quantified using Adobe Photoshop and presented as average percent healing of the original defect size (* $P < 0.05$) (Fig. 1B). Human ASCs when seeded in an acute wound showed over 60% healing within eight weeks postoperatively (light blue bars, Fig. 1B). In contrast, hASCs when seeded in a chronic wound showed less than 30% healing (dark blue bars, Fig. 1B) and empty defects healed less than 10% (white bars, Fig. 1B). Acute defects also healed more than scaffold alone control (see Supplemental Digital Content 3 -Figure 2 shows differences in osteogenic healing of a 4mm critical sized defect. (A) Micro-computed tomography of defect sites at stratified time points postoperatively. Defects were either left empty (first row), treated with an HA-PLGA scaffold (second row), or treated with a scaffold and hASCs immediately after the defect was created. (B) Micro-computed tomography of defect sites at stratified time points of an already established defect (defined as a defect left empty at 8 weeks postoperative). An established defect treated with a scaffold alone (first row). An established defect treated with a scaffold and hASCs (second row), a scaffold and hASCs pre-treated with PRP with re-injury of the bone edges (third row), and a scaffold and hASCs along with re-injury of bone edges (fourth row). Further priming of hASCs with PRP and more extensive debridements failed to enhance osteogenesis of established defects.

ASC healing of an acute and chronic calvarial defect by histological analysis

After 8 weeks of healing, animals were sacrificed and histological analysis was performed in order to confirm radiographic findings. Aniline blue staining was performed, in which osteoid appears dark blue (Fig. 2, top row). Histological analysis recapitulated findings by microCT: hASC engrafted scaffolds in an acute wound demonstrated significant Aniline blue positive bone formation within the defect site (Fig. 2B). In comparison, little bone formation was observed in either defects left empty or established defects treated with an hASC laden scaffold (Fig. 2A,C). Quantification of Aniline blue staining, determined by average pixels Aniline blue positive bone per low powered field, revealed a significant

increase in bone formation among hASC engrafted new defects only (Fig. 2D). Adjacent slides were stained with pentachrome (Fig 2, bottom row), in which bone stains yellow. Significant bone formation that completely bridges the defect site was again observed throughout hASC-treated acute defects. This was in marked contrast to the healing observed in the other groups, which showed little pentachrome staining.

Next, further histological stains were employed to examine further differences between acute and established hASC engrafted defects. Alkaline phosphatase staining was performed, in which the phosphatase activity turns histologic sections purple (Fig. 3, A–C, top row). Robust alkaline phosphatase staining was only present in hASC engrafted acute defects (Fig. 3B top row). In contrast, a near absence of alkaline phosphatase (ALP) activity was noted in hASC engrafted established defects (Fig. 3C top row). This difference in ALP was quantified in select slides, demonstrating a significant difference between the acute and established defect sites (Fig. 3D). Finally the anionic dye picrosirius red staining was performed, in which mature lamellar bone appears green under polarized light (Fig. 3A–C, bottom row). Lamellar bone was only observed in hASC engrafted new defects, but was not present in either hASC engrafted established defects or those defects left empty. These data suggest that hASCs engrafted HA-PLGA scaffolds can be successfully utilized to ossify an acute critical-size calvarial defect. However, an established calvarial defect of equal size does not ossify after hASC engraftment. Next, we attempted to look at the differences between an acute and established defect site in an effort to explain our findings that hASCs are capable of significant healing when placed in an acute but not an established defect site.

BMP expression is increased in an acute wound environment as compared to a chronic wound environment

Bone morphogenetic protein (BMP) signaling is of central importance in bone biology and skeletogenesis, and was first recognized for its ability to stimulate ectopic bone formation (14). We hypothesized that BMP expression may be upregulated in the acute wound environment, and thus performed *in situ* hybridization specific for several pro-osteogenic BMP genes (*Bmp2*, *Bmp4*, *Bmp7*; Fig. 4). For orientation Hematoxylin and Eosin (H&E) staining was performed (left column, Fig. 4). Histology was first obtained from an uninjured calvaria to demonstrate baseline *Bmp2*, 4 and 7 expression (Fig. 4, first row). Next, *in situ* hybridization of *Bmp2*, 4 and 7 was performed at stratified timepoints following acute injury (1–8 wks; Fig. 4, Rows 2–5) and 2 weeks after debridement of the established defect (Fig. 4, Row 6). Starting with *Bmp2* expression, we observed an intense signal at both 1 and 2 weeks postoperative. This signal largely dissipated by weeks 4 and 8 (Second column, Fig. 4). *Bmp4* and *Bmp7* *in situ* hybridization showed a similar pattern of distribution as well: each gene was strongly expressed at 1 and 2 wks post-injury (Fig. 4 Rows 3–4), but at 4 and 8 weeks post-injury expression staining notably decreased in intensity (Fig. 4, Rows 4–5). Even after re-injury of the old defect site, levels of *Bmp-2,4* and 7 failed to reach levels of the acute injury measured by *in situ* hybridization (Fig. 4 Row 6). These findings were further supported by qRT PCR which showed increased expression of *Bmp-2,4*, 7 and *Bmpr1b* two weeks after an acute defect compared to two weeks after debridement of an established defect (Fig. 4B). Collectively, these data suggested that the most commonly studied pro-osteogenic BMPs (*Bmp2*, 4 and 7) are highly expressed in the first two weeks following acute defect creation. In contrast, at 8 weeks following defect creation, or 2 weeks after debridement of the edges of an established defect, there was minimal BMP expression.

Platelet rich plasma induces growth and osteogenic differentiation of hASCs in vitro

Having observed differences in healing of an acute and established defect that correlated with BMP expression levels, we next set out to further understand the molecular basis behind these differences. One difference noted immediately following injury when

compared to eight weeks after injury is the amount of bleeding and the likely acute inflammatory milieu. Though care is taken to minimize bleeding during defect creation, bleeding was uniformly and consistently noted from the fresh cut edges of the bone. When hASCs were engrafted in an established defect less blood was noted following scar debridement. Drawing from these observations, we utilized activated platelet rich plasma (PRP) *in vitro*, to mimic an acute wound environment *in vivo*. The effects of platelet rich plasma on hASC *in vitro* proliferation and osteogenic differentiation was assessed (Fig. 5). PRP is a known mitogen in hASCs (15). Using BrdU incorporation and cell counting assays, we indeed found a significant mitogenic effect (Fig. 5 A and B). Next, we inquired as to the more relevant influence of PRP in ASC osteogenic differentiation.

By all markers examined, PRP induced a strong osteogenic response among hASCs. Alkaline phosphatase staining (Fig. 5C) and quantification (Fig. 5D) at 3d differentiation showed that hASCs treated with 5% platelet rich plasma had the most robust stain. Alizarin red staining and quantification at 7d differentiation showed that platelet rich plasma also had a significant pro-osteogenic effect hASC terminal differentiation (i.e. calcified matrix formation) (Fig. 5E and F). Results were verified by qRT-PCR, showing an overall increase in human osteogenic gene markers upon addition of PRP to ODM for three days of treatment (*OCN*, * $P < 0.05$) (Fig. 5G). Importantly, *BMP2* expression was also noted to increase in hASCs treated with 5% PRP, consistent with our *in vivo* findings by *in situ* hybridization (Fig. 5H).

Discussion

In many reconstructive surgeries for osseous defects, a fresh graft of autologous cancellous bone with hematopoietic bone marrow is considered to be an effective treatment option. The clinical application of autologous cancellous bone grafts, however, are often limited indicating the need for a more readily available material that is biologic in origin. ASCs have the ability to differentiate into osteogenic tissue in an acute calvarial defect,(4,7) likely in part due to the local wound milieu created by the surrounding osteoblasts and platelet derived growth factors.

Autologous blood derived PRP delivers growth factors that play an important role in the regulation of growth and development of tissue, and is in high concentrations at the site of acute bone defects. These factors include platelet-derived growth factor (PDGF), and insulin-like growth factor I (IGF-I), both of which have previously shown to stimulate osteogenesis of hASCs.(5) Other signaling pathways have been shown to be modulated by PRP, including TGF- β and BMP.(16,17)

Interestingly, we did not notice a dose dependency of PRP used. The concentration specific rather than dose-dependent effects of PRP may be best explained by comparing these data to previous studies that have focused on the individual cytokine components of PRP. For example, we previously examined the effects of TGF- β 1 on the osteogenic differentiation of hASCs.(6) In this study and in similarity to the effects of PRP, TGF- β 1 showed concentration specific effects rather than dose-dependency in many cases. In another related study, we examined the effects of PDGF in hASC osteogenic differentiation, another often cited cytokine component of PRP.(18) Again, dose dependency in the effects of PDGF on osteogenesis was not observed. Therefore, if the individual and predominant cytokines of PRP do not induce a dose-dependent increase in hASC osteogenesis when added alone, it seems reasonable as well that PRP itself may not show dose-dependency.

Others have demonstrated that PRP acceleration of bone fracture healing is due to changes in BMP-2 expression and activation.(16,17,19) We demonstrated that the wound environment of an acute calvarial defect expresses elevated transcripts of pro-osteogenic

BMPs and that this expression decreases after eight weeks healing. It is this environment of high endogenous pro-osteogenic BMP expression that likely can contribute to the improved osteogenic capacity of hASCs in an acute as compared to an established defect. A possible explanation for this increase in BMP expression lies in the high levels of TNF- α in an acute wound. TNF- α has been shown to promote osteogenic differentiation of human mesenchymal cells through NF- κ B mediated BMP-2 release.(20) Such levels of TNF- α are known to be elevated in an acute wound environment and TNF- α is a known component of PRP which might explain the osteogenic effect of PRP on hASCs. Future studies will attempt to further characterize the cytokine milieu present in an acute skeletal defect that is lacking in an established skeletal defect.

In this context, PRP may prove valuable for use in conjunction with hASCs to enhance osseous healing. Unfortunately, human PRP caused an inflammatory reaction in the mice injected with PRP (data not shown) and PRP priming in hASCs failed to enhance osseous healing (Supplemental Figure 2). Future studies that allow for a more controlled release of the factors in PRP such as BMP-2 may allow for healing of a chronic calvarial defect. Human ASCs are a readily available cell source capable of osteogenesis that, when paired with PRP, are capable of even greater osteogenic differentiation. Perhaps in a similar technique to bone grafts which are soaked in a blood soaked gauze prior to transplantation and stabilization, one can foresee an osteogenic scaffold seeded with hASCs soaked in PRP previously harvested from the patient which would eliminate the cross species reaction noted of treating mice with human PRP. Importantly, however, PRP contains numerous individual cytokines, which may vary in relative abundance from patient to patient.(21) The identification of the exact combination of cytokines among these factors that maximally induce osteogenic differentiation are the focus of future investigations.

Conclusions

Undifferentiated hASCs heal an acute calvarial defect. This, however, is not observed when hASCs are engrafted into an established calvarial defect. These data suggest that signaling pathways activated post-injury are critical for successful hASC mediated bony repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Differences in osteogenic healing of a 4mm critical sized defect. (A) Micro-computed tomography of defect sites at stratified timepoints postoperatively. Defects were either left empty (first row), treated with an HA-PLGA scaffold and hASCs immediately after the defect was created ('acute,' second row), or treated with a scaffold and hASCs eight weeks after the defect was created ('established defect,' third row, $n=5$ per group). (B) At 2, 4, 6 and 8 wks, healing as assessed by microCT was quantified using Adobe Photoshop, and presented as average percentage osseous healing of the original defect size. $N=5$ mice per group. A one-factor ANOVA was utilized, followed by a post-hoc student's t -test to assess significance. $*P < 0.05$.

Figure 2.

Histologic analysis of bone formation. After 8 wks, mice were sacrificed for histological analysis. From left to right, defects shown include those left empty (A), those treated with scaffold with hASCs immediately after the defect creation (B), or those treated with a scaffold with hASCs eight weeks after the defect creation (C). Stains include Aniline Blue (top) and Pentachrome (bottom). (D) At 8 wks, Aniline Blue positive bone per 2.5× field was quantified, $n=50$ slides per group, 5 animals per group. A one-factor ANOVA was utilized, followed by a post-hoc student's t -test to assess significance. $*P < 0.05$. Boxes represent defect site at 20X magnification.

Figure 3.

Histologic analysis of osteogenic activity. From left to right, defects shown include those left empty (A), those treated with an hASC engrafted scaffold immediately after the defect creation (B), or those treated with an hASC engrafted scaffold eight weeks after defect creation (C). Stains include Alkaline Phosphatase (top) and Picrosirius Red (bottom). (D) At 8 wks, average Alkaline phosphatase positive bone per 2.5× field was quantified, $n=5$ slides per group, 5 animals per group. A one-factor ANOVA was utilized, followed by a post-hoc student's t -test to assess significance. $*P < 0.05$. Boxes represent defect site at 20X and 10X magnification.

Figure 4.

An acute calvarial defect site expresses increased levels of pro-osteogenic BMPs immediately following injury. These high levels of BMP expression decrease over time, and re-debridement of an established defect does not cause the same elevation in BMP expression. (A) *In situ* hybridization for *Bmp2*, *Bmp4*, and *Bmp7* at 1, 2, 4, and 8 weeks postoperatively in an acute defect. The bottom row shows BMP expression 2 weeks after re-debridement of an established defect wound. From left to right, column 1 shows an H & E stain of the defect site for orientation. Column 2 demonstrates *Bmp2* expression by *in situ* hybridization. The top row shows the uninjured side as a baseline comparison. Column 3 and 4 show *Bmp4* and *Bmp7* expression, respectively. (B) *qRT-PCR* comparing BMP

expression levels 2 weeks postoperatively (acute defect) to BMP expression levels 2 weeks following debridement of an already established defect. * $p < 0.001$)

BrdU Incorporation

Alizarin Red

Alizarin Red Quantification

Figure 5.

Effects of Platelet Rich Plasma (PRP). (A) BrdU incorporation assays at days 1–7. (B) Cell counting assays at days 1–7. (C) Alkaline Phosphatase stain and (D) alkaline phosphatase quantification after 3 days differentiation. (E) Alizarin Red stain and (F) quantification at 7 days. (G) Gene expression of human *OCN* and (H) *BMP-2* in hASCs after three days of osteogenic differentiation with or without PRP. $n=3$ wells for all assays, $*P < 0.05$. A two-tailed student's *t*-test was performed to assess significance.