Skeletal Trauma Generates Systemic BMP2 Activation That Is Temporally Related to the Mobilization of CD73+ Cells

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ABSTRACT: The relationship between BMP2 expression and the recruitment of skeletogenic stem cells was assessed following bone marrow reaming. BMP2 expression was examined using transgenic mice in which β-galactosidase had been inserted into the coding region of BMP2. Stem cell mobilization was analyzed by FACS analysis using CD73, a marker associated with bone marrow stromal stem cells. BMP2 expression was induced in endosteal lining cells, cortical osteocytes and periosteal cells in both the reamed and in contralateral bones. BMP2 mRNA expression in the reamed bone showed an early peak within the first 24 h of reaming followed by a later peak at 7 days, while contralateral bones only showed the 7 days peak of expression. FACS analysis sorting on CD73 positive cells showed a 50% increase of these cells at 3 and 14 days in the marrow of the injured bone and a single peak at 14 days of the marrow cell population of the contralateral bone. A ~20% increase of CD73 positive cells was seen in the peripheral blood 2 days after reaming. These data showed that traumatic bone injury caused a systemic induction of BMP2 expression and that this increase is correlated with the mobilization of CD73 positive cells. © 2013 The Authors. Journal of Orthopaedic Research Published by Wiley Periodicals, Inc. on behalf of the Orthopaedic Research Society. J Orthop Res 32:17–23, 2014.

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Skeletal trauma initiates the recruitment of mesenchymal stem cells (MSCs) that contribute to regeneration of the injured bone. Studies have further shown that bone trauma will lead to systemic recruitment of osteogenic progenitors or stem cells that contribute to the bone healing based on the presence of CD73 positive plastic adherent mesenchymal stem cells that are found in the circulation after trauma.[1–3] BMP2 has been shown to be a necessary morphogenetic factor that promotes the differentiation of these skeletogenic stem cells at the site of trauma.[4,5] However, the molecular mechanisms by which stem cells are recruited to the injured site, the degree to which these cells are local or systemic in origin and whether BMP2 contributes in a systemic manner to the mobilization and or recruitment of stem cells to the injured site is not well understood.

Mice containing a bacterial artificial chromosomes (BAC) transgene in which β-galactosidase had been inserted into the coding region of BMP2 were used in a prior study to assess the cellular expression of BMP2 during the bone formation that was induced by distraction osteogenesis. This study showed that BMP2 expression was induced in smooth muscle and vascular endothelial cells of arteries and veins and capillaries, as well as hypertrophic chondrocytes and osteocytes during bone formation. These data further showed that the majority of BMP2 expression was in the vascular tissues within the musculature surrounding the distraction gap, and not in the vessels within the bone itself.[6] Contrary to our expectation, these results did not show BMP2 expression in any of the mesenchymal cells that were initially recruited into the repair site. Such results therefore suggest that within this model of primarily intramembranous bone regeneration and repair that the source of BMP2 that promoted skeletogenic stem cell differentiation was paracrine in nature and most likely came from the circulation. In other studies, we have shown that BMP2 was necessary for bone repair in a model of intramembraneous bone regeneration that was induced by medullary reaming.[4]

In the current study, the cellular origins of BMP2 expression were examined after medullary reaming since this model of bone repair induces an injury that is only localized within the bone, and therefore the trauma should have no effect on the muscle or vessels outside of the bone. The data that is presented here show that this more localized bone injury induced the expression of BMP2 locally in osteocytes, cells in the periosteum, and cells on the endosteal surface primarily under the growth plate. Interestingly reaming also induced BMP2 expression in similar populations of cells in other non-injured bones but at lower levels. We also showed that localized bone injury increased the numbers of CD73 positive cells within the marrow throughout the animal and these cells appeared in the circulation after injury.

MATERIALS AND METHODS
Materials
The BMP2 BAC transgenic mouse line contained ~185 kb 5′ to the BMP2, the entire ~11 kb coding region of the gene in which the second exon had been exchanged for the β-galactosidase gene and ~42 kb of 3′ sequence.[7] All transgenic mice were bred on site, and both heterozygotes and homozygotes containing the transgenes were used for our studies.

All reagents for the PCR analysis were from Applied Biosystems (Carlsbad, CA), and plate assays were read on an ABI 7700 Sequence Detector (Applied Biosystems). Pertinent sequence information and amplicon sizes for all Taqman gene expression assays are available at https://products.appliedbiosystems.com/ab/en/US/adirect/ab.

Experimental Design and Surgical Procedure

All animal studies were carried out under an approved Boston University Medical School Institutional Animal Care and Use Committee (IACUC) protocol. Male mice of ∼15 weeks of age weighing between 25 and 35 g were used for these studies. A previously described model of tibia reaming for surgically ablating the marrow was used for these studies.[4,8,9] Wild-type C57 BL/6J or *BMP-2-Lac-Z* mice were anesthetized with 4% isoflurane and their right hind legs were shaved and prepped with betadine solution. A short longitudinal incision was made through the skin and the medial half of the patellar tendon. With the knee in flexion, a 0.5 in. 25 gauge needle was used to create a starting portal centromedially in the tibial plateau. Sequential reaming of the tibial medullary space extending to the level of the middiaphyseal bow in the bone was performed using 30, 27, 25, and 23 gauge needles, respectively. The skin was then closed in a single layer fashion using 4–0 vicryl suture. Closed single transverse femur fractures were generated as described for mouse femur.[10] Animals were euthanized by CO2 asphyxiation.

Fluorescence-Activated Cell Sorting (FACS)

For cell sorting studies bone marrow cells were derived from the reamed limb and the contralateral tibias from the same mice at 12 h, 1 day, 3 days, 7 days, 14 days, and 21 days following surgery or from separate naïve control animals that were unoperated. Proximal and distal condyles were removed from each tibia and the marrow space was flushed with 5 ml αMEM-media to collect all cells contained in the marrow cavity. Cells were then concentrated by centrifugation and resuspended in 1 ml αMEM-media. The cells were then divided into aliquots and each cell batch was stained using PE-labeled antibodies for CD29, CD45, CD73, CD105, Sca-1, and C-Kit. Antibodies were chosen specifically to differentiate MSCs (CD105, Sca-1, CD73) from the hematopoietic lineages (CD29, CD45, and c-Kit). Flow cytometry was performed for each marker or for PE Mouse IgG1, κ Isotype control (BD Biosciences, Bedford, MA, USA). Reactions with each PE monoclonal antibody were done for 30 min on ice. Sorting was carried out using a FACS-Calibur machine (BD Biosciences). For FACS of cells in the peripheral blood, the animals were bled by cardiac puncture following euthanasia at 2 and 6 days after surgery. FACS analysis of peripheral blood specimens was carried out after the red blood cells were removed using a RBC lysis buffer (BD Bioscience) according to manufacturer's protocol for whole blood. In these studies cells were reacted after RBC lysis with PE monoclonal antibody for CD73 and FITC monoclonal antibodies for CXCR4. Analysis was performed both as individual samples for CD73 and CXCR4, respectively, and a third vial with combined PE-CD73 and FITC-CXCR4 for co-expression analysis. The data were analyzed by using BD Cellquest Pro v5.2 software (BD Biosciences). All cell populations were prepared from the medullary space or blood from $N = 3$ animals per experimental group at each time point and FACS analysis was repeated at least three times.

RNA Isolation and Quantitative Real-Time RT-PCR

RNA was prepared from the same three experimental groups as described above. Samples were collected at time points 2 h, 12 h, 24 h, 3 days, 7 days, 14 days, and 21 days after surgery. After euthanasia specimens were prepared by removing the distal cartilage condylar surfaces of the tibia and the bone was cut at the middiaphyseal bow. These segments of whole bone tissues were collected into liquid nitrogen and stored at −80°C until they were used for RNA extraction. The RNA extraction and quantitative real-time RT-PCR were carried out as previously described.[11] Analysis of mRNA expression was carried out on replicate pools ($N = 3$ mice per pool) of mRNAs and individual assessments were done three times on each pool. For cell culture experiments, RNA samples were measured from the average value of three separate cell preparations composed of triplicate samples ($N = 9$). For the in vivo studies expression values of target genes were normalized to bones from unoperated controls while for cell culture experiments all samples are expressed as a ratio of cultures that were untreated with BMP2. All mRNA levels were normalized to β-actin and the fractional cycle number at which the fluorescence passes the fixed threshold (C_t values) was used for quantification by using a comparative C_t method.

Bone Marrow Stromal Cultures

Bone marrow stromal cell preparations were made as previously reported.[12] All experiments were performed with at least three separate cell preparations, and all measurements from any given set of cell preparations were carried out with three replicates per group. MSC cells were plated to reach an initial confluence of approximately 50% followed by media change every other day until day 12. At day 12, the MSCs cultures were divided into two groups, one treated with α-MEM media (10% FBS) supplemented with rhBMP2 to a concentration of 100 ng/ml, and the other were only treated with α-MEM media (10% FBS). Media was changed every other day. Collection of cultures for further FACS-analysis was performed at time points 24 h, 48 h, 72 h, 96 h, and 120 h after start of treatment.

Histology and Staining for β-Galactosidase

Staining for β-galactosidase was performed on freshly dissected long bones free from soft tissues. All specimens were fixed for 75 min at 4°C in 4% paraformaldehyde and then washed 3× at 20 min increments in 0.1 m sodium phosphate buffer (pH 7.3), 2 mm MgCl, 0.01% deoxycholate, 0.02% Nonidet P-40. Following the fixation and wash, β-galactosidase staining was performed in the wash buffer supplemented with 1 mg/mL X-gal solution (GIBCO-BRL, Gaithersburg, MD), 4 mm K3Fe(CN)6, 4 mm K4Fe(CN)6 3 H_2O, and 0.1 m Tris (pH 7.4) for 24 h at room temperature. Finally, the bones were washed 3 × 10 min in wash buffer before photography and analysis in cases where whole bone staining was to be assessed. Subsequently stained specimens were then placed into fresh 4% paraformaldehyde for 48 h for final fixation for further histological processing. After fixation, samples were decalcified in (14%, w/v), ethylene diamine tetra acetic acid (EDTA) (BioProducts, Boston, MA) for 2 weeks. Once the specimens were decalcified, they were embedded in paraffin and sectioned at 5 μm in the sagittal plane. Sections for plain histology were cleared and stained with hematoxylin and eosin.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Statistics were overall calculated with Unpaired Student's *t*-test (two-tailed) or as indicated and described elsewhere in the results section. A *p*-value <0.05 was considered statistically significant.

RESULTS

Reaming Induces Systemic BMP2 Expression

The first studies that were performed focused on the cellular expression of BMP2 during intramedullary intramembranous bone formation that was induced by reaming. Mice containing the BAC transgene in which β-galactosidase had been inserted into the coding region of BMP2 were used in these studies to assess BMP2 expression (Fig. 1). At 7 days post-surgery gross morphology for both the reamed and the contralateral bones demonstrated that staining was detectable at high levels throughout the whole bone. As an initial control, the uninjured contralateral tibiae from the reamed mice, were assessed. However, surprisingly these bones also showed a marked increase in BMP2 expression although the staining was less than the reamed bone (Fig. 1A). In order to determine if the staining of the contralateral tibiae was detecting a base line level of BMP2 expression in the bones, a third control was assessed, by staining the tibiae from mice in which no reaming had been carried out. These bones showed only a very light staining at the joint surfaces, which led to the conclusion that there was a systemic increase in BMP2 expression in the skeleton following a local trauma in an isolated bone organ.

As a further experiment to determine if bone trauma in general induced systemic activation of BMP2, femur fractures were carried out and the contralateral femurs and tibias of these animals were examined at 7 days postfracture. These results further confirmed the systemic nature of the BMP2 activation in response to a trauma but in the context of an endochondral bone formation mechanism (Fig. 1A).

Histological analysis was next performed to identify the cell types that were expressing BMP2. These
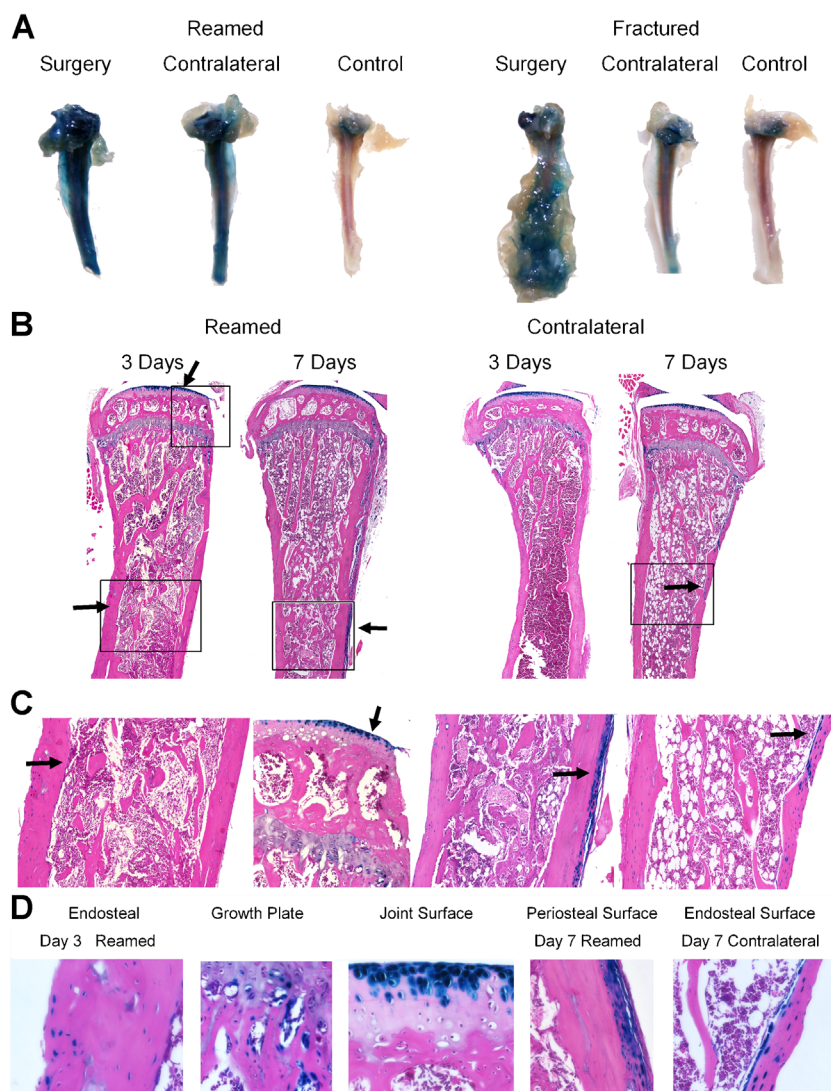

Figure 1. Systemic induction of BMP2 expression in response to skeltal trauma. (A) Gross assessment of BMP2 expression in traumatized tibias 7 days post-surgery (reamed = tibia undergoing surgery, contralateral = tibia from the contralateral leg of the same animal that underwent surgery, control = tibia from Naive mouse that received no surgery), BMP2 expression is indicated by the blue staining for β-galactosidase expression of the transgene. (fractured = surgery, contralateral and control are as denoted for the reamed samples. (B–D) Histological analysis of tissues and cells expressing BMP2. Areas of higher magnification seen in C (100×) and D (400×) are boxed in panel B. Anatomical sites of the sample and bone surface from which the micrographs are obtained is denoted in the figure. Arrows are placed to highlight specific cell types that show strong expression and highlight the presence of BMP2 expression within osteocytes in cortical bone towards the periosteal surface and cortical lining cells on the endosteal surface. At all time points, all specimens show strong basal BMP2 expression in cells throughout the hypertrophic growth plate zone and chondrocytes lining the joint surfaces (B and C: second from left and D: growth plate and joint surface).

studies showed that at 3 days after reaming, osteocytes within the cortical bone towards the periosteal surface and cortical endosteal lining cells were expressing the highest levels of BMP2. As the repair progressed at 7 days postreaming, there was very intense staining for BMP2 expression within the periostieum which showed a clear thickening in areas adjacent to where bone formation was taking place. In contrast, most of the reactivity in the contralateral bone was seen in cortical osteocytes closer to the endosteal surface. Interestingly, no cells were stained in the intramedullary areas in which new mesenchymal stem cells are being recruited into areas of newly formed osteoid. Within all the specimens, cells throughout the hypertrophic zone of the growth plate and chondrocytes lining the joint surfaces showed very strong reactivity which appear to be basally expressing BMP2 at all times (Fig. 1B–D).

Temporal Relationship Between BMP2 and CXCR4/SDF-1 mRNA Expression

The systemic nature of BMP2 expression after trauma led us to hypothesize that there would be increased hematopoietic and/or mesenchymal stem cell mobilization throughout the skeleton. In order to test this hypothesis, we chose to assay the temporal mRNA expression of BMP2 in relation to the CXCR4 chemokine receptor and its ligand, stromal derived factor 1 (SDF-1), since their expression has been shown to be associated with the systemic mobilization of mesenchymal and hemopoietic stem cells[13,14] (Fig. 2). These results showed that BMP2 expression in the reamed bone had a bimodal pattern with peaks at 12 h (3.5-fold increase) and 7 days (2.6-fold increase; Fig. 2). The expression in the contralateral tibias showed a different bimodal temporal pattern of expression with peaks at 2 h (2.2-fold increase) and 14 days (2.2-fold increase). The analysis of the CXCR4 receptor in the reamed limb showed an almost identical profile both in its scale of induction and temporal profile to that of BMP2 while in the contralateral limb the early peak showed good correspondence at later times, CXCR4 expression peaked a week after BMP2 expression. Interestingly, CXCR4 showed divergent expression from BMP2 at 21 days after reaming and showed increased expression in both the contralateral and reamed bones. SDF-1 expression showed good correspondence to BMP2 expression in the injured limb only for its later peak of expression, while SDF-1 expression was similar to that of its receptor in the contralateral limb. It is also interesting to note that overall the expression of SDF-1 was greater in the uninjured limb than in the injured limb. As a control, TGF-β1 was examined in this analysis, since previous studies had shown that it was involved in the local recruitment of mesenchymal stem recruitment during the coupled remodeling of bone.[15] It is interesting to note that unlike CXCR4 and SDF-1 expression which showed systemic induction after trauma, TGF-β1 was induced immediately after reaming (2 h) in the uninjured limb and then at the 7 days peak concurrent with the initiation of coupled remodeling.
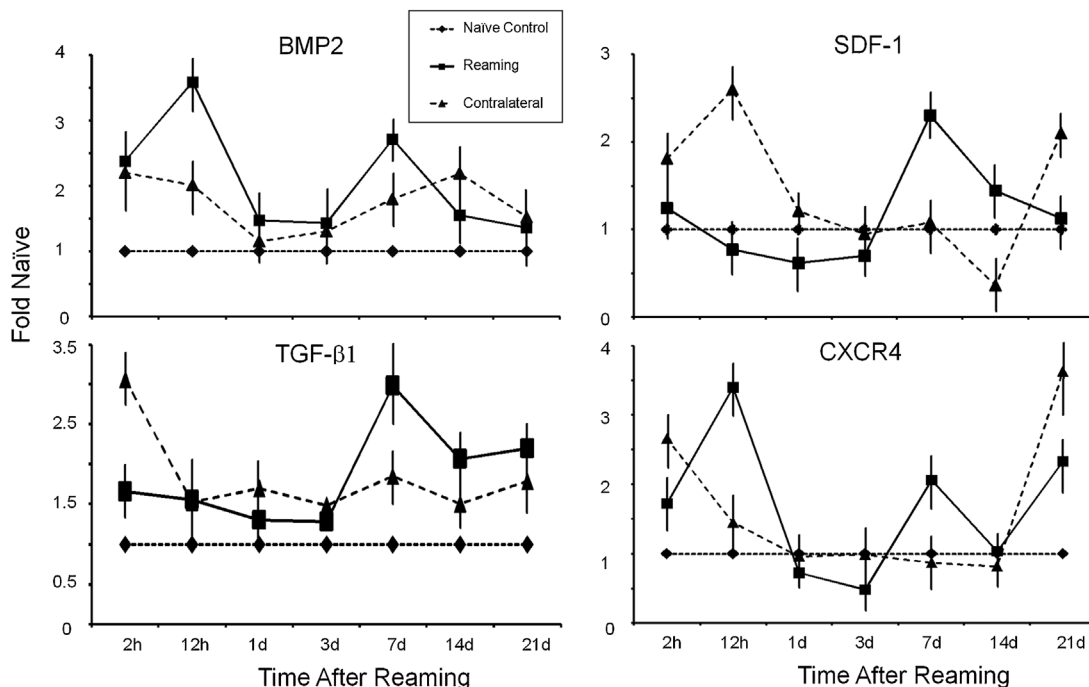

Figure 2. Steady state mRNA expression of morphogens and markers associated with stem cell mobilization whole bone segments from the regions that had been reamed or comparable regions from contralateral and control bones as described in Materials and Methods Section, were used for these studies. qRT-PCR analysis was made for RNAs from (Reamed) bones compared to the (Contralateral) of the traumatized mice and from uninjured mice (Naïve Control) tibia. Data are displayed as fold change versus Naïve Control set as base line (=1). Time after surgery and the nature of each mRNA that was examined is denoted in the figure.

Fluorescence-Activated Cell Sorting Analysis of Marrow Stromal Cell Populations

In order to determine how reaming altered the various cell populations in the bone marrow FACS analysis was next carried out. A series of surface markers that have been associated with mesenchymal (CD73, CD105, Sca-1) and hemopoietic stem cell populations (CD29 and C-Kit) were used with the general marker for hematopoietic/myeloid cells (CD45) used as an additional control.[16,17] The overall percentage of cells expressing CD29, CD45, and c-Kit showed no change in either the reamed or uninjured bones. In contrast, the cell populations that were Sca-1 and CD105 positive showed the same percentage and temporal patterns of
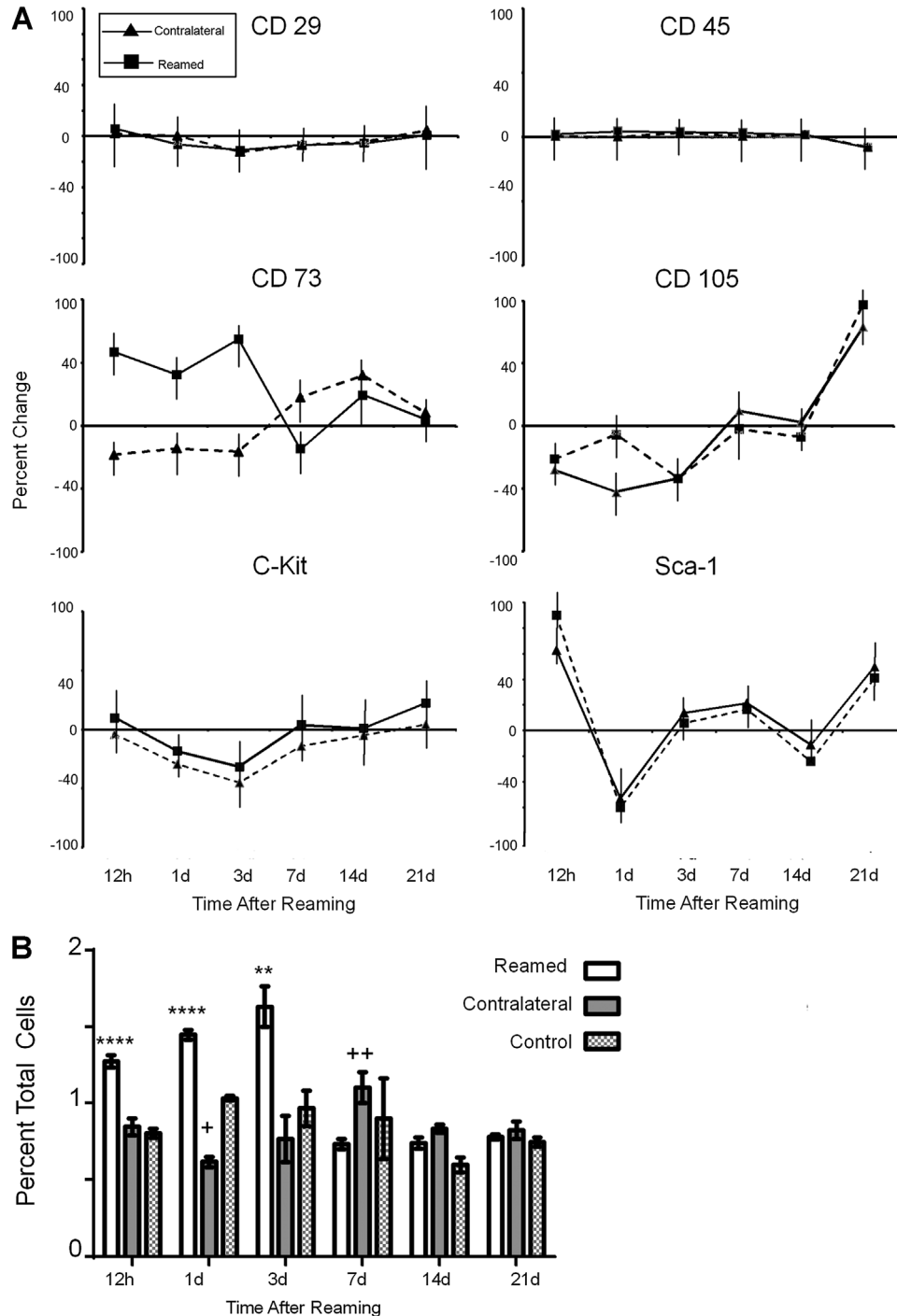

Figure 3. Change in marrow cell populations following reaming. (A) Percentage change in marrow cell populations expressing various cell surface markers as determined by FACS analysis. Cell markers are as denoted in the figure and are from cells collected from bone marrow of reamed and contralateral tibias. Data are presented as percent change compared to MSCs from naïve control bone (set as base line = x-axis). (B) Absolute percentage CD73 positive cells found within the marrow of contralateral reamed and naive control bones at various times after surgery (* = reamed vs. naïve control, $+$ = contralateral vs. naïve control). $^{****}p \leq 0.0001$, **,$++$ $p \leq 0.01$, $+p \leq 0.05$).

increases in both the reamed and uninjured bone, although the temporal patterns were very different between two populations that expressed these markers. The most interesting result however was for CD73 positive cell populations, compared to the other markers. In the reamed bone, CD73 showed a marked increase (50–60%) at 12 h, 24 h and 3 days, followed by a drop below baseline levels at 7 days after surgery and then a small increase at day 14. At the same time, in the uninjured bone this marker showed a decrease of ~25% compared to baseline at 12 h, 24 h and 3 days, followed by a 50% increase throughout the rest of the time course (Fig. 3).

The reciprocal relationship for the percentages of CD73 positive cells in the reamed and uninjured bones suggested that this population of cells might be recruited from the uninjured bone to the site of injury. In order to determine if reaming mobilized this population of cells into the circulation, the percentage of cells that sorted on CXCR4 or CD73 were next examined in the peripheral blood (Fig. 4). These results showed that cells positive for these two surface markers were indeed elevated in peripheral blood after injury consistent with prior results showing that CD73 and CXCR4 cells are mobilized into the circulation after fracture.[17]

BMP2 Increases the Percentage of CD73 Positive Cells in MSC Cultures

To investigate the effects of BMP2 on MSC CD73 expression, we performed in vitro experiments of rhBMP2 treatment of primary cultures of MSCs followed by FACS analysis at time points 24, 48, 72, 96, and 120 h after start of treatment. The analysis revealed that the number of CD73 positive cells increased following treatment of the cells with media supplemented with rhBMP2 compared to the control group which only received ordinary media treatment. The results showed that CD73 expression was significantly increased at the 48 h (43%, $p = 0.0072$), 72 h (49%, $p = 0.0002$) and 96 h (21%, $p = 0.0034$) time points whereas a decrease versus control was present after 120 h (-30%, $p = 0.0018$; Fig. 5).

DISCUSSION

These data showed that skeletal trauma induces a systemic osteogenic response, in both non-traumatized bones and bone marrow, which contributes to the healing of the injured bone. We show an increased systemic expression of BMP2 following trauma, suggesting a much wider role for this protein than the local actions that are suggested by most previously reported models. It is also interesting to note the recent demonstration of a regulatory element in the BMP2 gene that autonomously controls BMP2 expression in mesenchymal cells has the potential to activate its expression in response to pro-inflammatory signals, oxidative stress, and metabolic changes.[18] This type of response is also consistent with data showing that increasing BMP2 levels will autologously up-regulate its own expression during the progression of osteogenic differentiation within bone marrow stromal cell cultures.[4,12] Finally it is of interest to note that the current data showing the distribution of endogenous BMP2 expression within osteocytes and lining chondrocytes in joints is consistent with our own prior studies[6] and those of others.[19]

A second question that was addressed in this study was the relationship between BMP2 expression and the systemic recruitment of MSCs from uninjured bone marrow. It is interesting to note that previous studies have shown that both direct trauma to the bone marrow or systemic blood loss lead to a systemic osteogenic response.[20] More recent reports have also suggested that bone trauma will lead to systemic recruitment of osteogenic progenitors or stem cells that contribute to the bone healing.[1,2] In this regard our current data are consistent with prior studies that have shown the mobilization of cells positive for both CD73+ or CXCR4 from the bone marrow after skeletal surgery.[2,21] Our data are also consistent with other studies that have shown that rhBMP2 treatment increases CD73+ cells when added to MSCs grown in vitro.[22] Our finding that CD73+ is increased following trauma in vivo is also of great interest since it has recently been shown that MSCs grown in vitro have the ability to home to the traumatized site when injected intravenously if they co-express CXCR4.[2] These MSCs were derived from bone marrow aspirate depleted of hematopoietic cells, and when grown in vitro >90% were CD73+ CD29+, and 67% CD105+. Since our data show a decrease of both CD29+ and
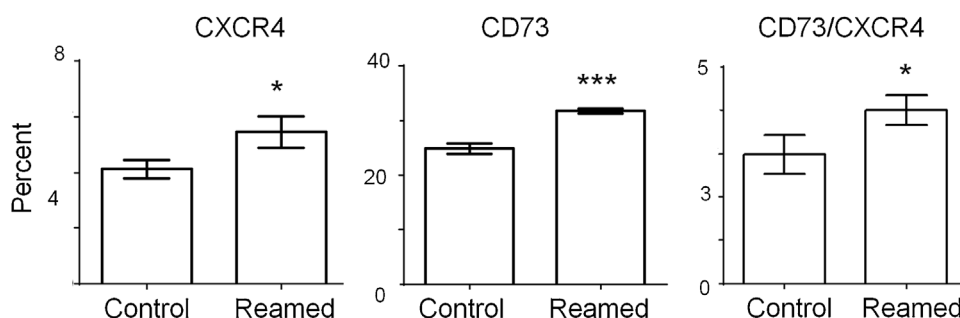

Figure 4. Systemic mobilization of CD73 and CXCR4 positive cells after surgery FACS analysis of CD73 and CXCR4 positive cells in the peripheral blood after reaming compared to naïve controls. Statistically significant increases were found for CD73 and CXCR4 when measured individually, as well as that population of cells that co-expressed both markers (*$p \leq 0.05$, ***$p \leq 0.001$).
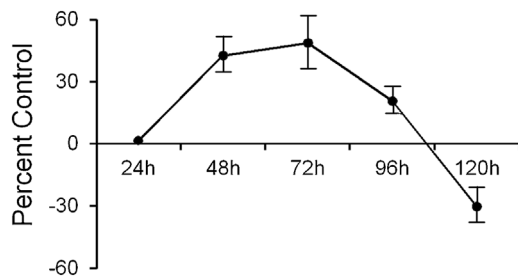
Figure 5. CD73 is induced by BMP2 within MSC. Effects of rhBMP2-treatment on the expression of CD73 within primary cultured MSC in vitro followed by FACS analysis. CD73 positive MSCs compared to the control (unsupplemented αMEM treatment) at 48 h (43%, $p = 0.0072$), 72 h (49%, $p = 0.0002$) and 96 h (21%, $p = 0.0034$) whereas a decrease versus control was present after 120 h (-30%, $p = 0.0018$).

CD105+ cells, but a marked increase in CD73+ cells following trauma in vivo, this suggests that CD73 is a potential key cell marker in finding the original MSC needed for bone regeneration. A primary deficiency of our current study however, was that the majority of the FACS analysis was carried out on single markers, while co-sorting on the multiple markers shown to be associated with expressed by MSCs would have provided a much better definition of this cell populations.

In conclusion, these findings are novel in that they show the systemic activation of BMP2 in the skeletal system in response to skeletal trauma and may have important implications for the use of BMPs in clinical practice. They also provide confirmatory data to the observation of the mobilization of CD73+ cells after traumatic skeletal injury which may be related to the systemic induction of BMP2.

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