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Journal of Cellular Biochemistry 112:2748–2758 (2011)

Journal of Cellular Biochemistry

Sensory Nerve Induced Inflammation Contributes to Heterotopic Ossification

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ABSTRACT

Heterotopic ossification (HO), or bone formation in soft tissues, is often the result of traumatic injury. Much evidence has linked the release of BMPs (bone morphogenetic proteins) upon injury to this process. HO was once thought to be a rare occurrence, but recent statistics from the military suggest that as many as 60% of traumatic injuries, resulting from bomb blasts, have associated HO. In this study, we attempt to define the role of peripheral nerves in this process. Since BMP2 has been shown previously to induce release of the neuroinflammatory molecules, substance P (SP) and calcitonin gene related peptide (CGRP), from peripheral, sensory neurons, we examined this process *in vivo*. SP and CGRP are rapidly expressed upon delivery of BMP2 and remain elevated throughout bone formation. In animals lacking functional sensory neurons ($TRPV1^{-/-}$), BMP2-mediated increases in SP and CGRP were suppressed as compared to the normal animals, and HO was dramatically inhibited in these deficient mice, suggesting that neuroinflammation plays a functional role. Mast cells, known to be recruited by SP and CGRP, were elevated after BMP2 induction. These mast cells were localized to the nerve structures and underwent degranulation. When degranulation was inhibited using cromolyn, HO was again reduced significantly. Immunohistochemical analysis revealed nerves expressing the stem cell markers nanog and Klf4, as well as the osteoblast marker osterix, after BMP2 induction, in mice treated with cromolyn. The data collectively suggest that BMP2 can act directly on sensory neurons to induce neurogenic inflammation, resulting in nerve remodeling and the migration/release of osteogenic and other stem cells from the nerve. Further, blocking this process significantly reduces HO, suggesting that the stem cell population contributes to bone formation. J. Cell. Biochem. 112: 2748–2758, 2011.

KEY WORDS: HETEROTOPIC OSSIFICATION; NEUROGENIC INFLAMMATION; BONE MORPHOGENETIC PROTEIN TYPE 2; NEURAL CREST STEM CELLS

Bone morphogenetic proteins, named for their original isolation from ground bone [Urist, 1965], are a family of factors involved in patterning of the bone, vessels [Smadja et al., 2008], nerves [Anderson et al., 2006], blood [Bhatia et al., 1999], and, most recently, fat [Sottile and Seuwen, 2000; Tseng et al., 2008] of the early embryo. The BMPs appear to serve a similar role in the adult, where they have been shown to regulate the expansion and differentiation of progenitors for these same tissues [Yanagita, 2009]. One of the best studied of the BMPs, BMP2, is a highly conserved protein that has been shown to be the only common link between the various forms of heterotopic ossification (HO) [Kaplan et al., 2009a; Kaplan et al., 2009b; Shore and Kaplan, 2010].

HO, or bone formation at non-skeletal sites, is a disorder resulting from traumatic injury [Vanden Bossche and Vanderstraeten, 2005],

Additional supplementary information may be found in the online version of this article.

Grant sponsor: US Department of the Army; Grant numbers: DAMD W81XWH-07-1-0214, DAMD W81XWH-07-1-0215; Grant sponsor: NBIB; Grant number: EB005173; Grant sponsor: NIH; Grant number: T-32 HL092332-06; Grant sponsor: American Heart Association; Grant number: AHA 10815339F; Grant sponsor: Kirschstein-NRSA; Grant number: T32 HL092332-08.

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Received 1 June 2011; Accepted 3 June 2011 • DOI 10.1002/jcb.23225 • © 2011 Wiley-Liss, Inc.
Published online 15 June 2011 in Wiley Online Library (wileyonlinelibrary.com).

with approximately 5% of all traumatic injuries leading to HO. HO has been linked specifically to the nervous system, as it appears after injury of the central nervous system (CNS) [Baird and Kang, 2009]. Maintenance of skeletal bone has been demonstrated to involve the CNS, through hypothalamic control of a number of neuroendocrine factors believed to directly regulate osteoblast function [Fu et al., 2005; Takeda and Karsenty, 2008; Ducy and Karsenty, 2010; Oury et al., 2010; Shi et al., 2010]. However, it is unclear that this mechanism results in the formation of ectopic bone. Some of the most compelling data linking HO formation to changes in the peripheral (PNS) or central nervous system come from recent statistics from the military, which suggest that 60% of all combat injuries have associated HO [Forsberg et al., 2009]. This is presumably because these are mostly blast or burn injuries, both having a significant impact on the PNS and CNS.

Little is known about the causes of HO, beyond the common link to changes in BMP signaling. BMP2 has been shown to be elevated upon muscle injury and changes in blood flow, and BMP2 is released during bone injury [Beiner and Jokl, 2002; Johnson et al., 2006; Kwong and Harris, 2008; Sucosky et al., 2009]. Recently, BMP2 has also been shown to directly induce release of neuroinflammatory proteins from sensory neurons. Studies revealed the rapid up-regulation of substance P (SP) and calcitonin gene related peptide (CGRP) in sensory neuron cultures, after addition of recombinant BMP2 [Bucelli et al., 2008].

The afferent, sensory fibers of the PNS release SP and CGRP leading to the induction of neuroinflammation. These sensory neurons are stimulated by noxious mechanical, thermal, or chemical stimuli, providing feedback on pain and temperature [Schaible et al., 2005], both locally and centrally through electrical signaling. The vanilloid (capsaicin) receptor TRPV1 (transient receptor potential cation channel V1) is a nociceptive ion channel located on sensory nerve endings that is activated by some of these noxious stimuli and involved in the mediation of pain [Szallasi et al., 2007; Khairatkar-Joshi and Szallasi, 2009]. Capsaicin, the compound in hot chili peppers, is one chemical stimulus, which can activate this channel, causing it to open, leading to an influx of calcium and sodium ions into the sensory neuron and triggering its depolarization. At normal levels, capsaicin binding transmits the sensation of pain. However, high doses of capsaicin lead to a massive influx of ions, resulting in death of sensory neurons expressing TRPV1. TRPV1 sensory neurons have been linked to a number of activities involving tissue regeneration and maintenance [Razavi et al., 2006], including repair of bone fractures, which were shown to be significantly inhibited when these nerves were ablated with capsaicin [Apel et al., 2009].

Morrison et al. [1999] isolated cells indistinguishable from neural crest stem cells from fetal peripheral nerves. Recently [Adameyko et al., 2009], such cells have been shown to be the origin of melanocytes in the skin in the adult. In addition, the bone tumor Ewing's sarcoma is composed of poorly differentiated cells that bear no relationship to mesenchymal cells, but show a definite relationship to neural crest stem cells [Cavazzana et al., 1987; Hu-Lieskovan et al., 2005]. This, coupled with the fact that some of the bones of the skull have been shown, using tracking techniques, to originate from the neural crest [Jiang et al., 2002], opens the question of whether the nerve may be an alternative niche for osteogenic progenitors. We provide evidence in these studies that suggest that the local peripheral nerves may indeed be an additional site for such progenitors.

We developed a model of rapid, de novo bone formation, or heterotopic ossification, which relies on expression of physiological levels of BMP2 at the site of bone formation [Olmsted-Davis et al., 2002; Gugala et al., 2003; Fouletier-Dilling et al., 2005; Fouletier-Dilling et al., 2007]. Upon delivery of adenovirus-transduced cells expressing BMP2 within the muscle, a cascade of endochondral bone formation occurs over 7 days, resulting in mature bone and bone marrow [Olmsted-Davis et al., 2007; Dilling et al., 2010]. Using this heterotopic bone assay, we reliably produce mature bone when cells transduced with BMP2 adenovirus are delivered, but not when cells transduced with an empty cassette, control adenovirus are delivered, showing this bone formation is linked to BMP2. In addition, we have previously found that the transduced cells do not incorporate into any structures of the newly forming heterotopic bone and are only serving to deliver the BMP2 [Gugala et al., 2003; Fouletier-Dilling et al., 2007]. In the studies described here, we have used this model to elucidate the manner in which peripheral nerves participate in HO.

MATERIALS AND METHODS

CELL CULTURE

A murine C57BL/6-derived fibroblast cell line was grown in α modified essential medium (α-MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B and cultured, as previously described [Dilling et al., 2010]. All cell types were grown at 37°C and 5% CO_2 in humidified air. The mouse fibroblast cell line was used only as a delivery cell for BMP2, and experiments repeated with multiple fibroblast cell types have yielded the same pattern of bone formation [Gugala et al., 2003; Olabisi et al., 2010].

ADENOVIRUSES AND CELL TRANSDUCTION

Replication defective E1–E3 deleted first generation human type 5 adenovirus (Ad5) were constructed to contain cDNAs for BMP2 in the E1 region of the virus [Olmsted-Davis et al., 2002] or did not contain any transgene in this region, Adempty. Mouse fibroblasts (1×10^6) were transduced with Ad5-BMP-2 or Ad5-empty control virus at a concentration of 5,000 vp/cell with 1.2% GeneJammer, as described previously [Fouletier-Dilling et al., 2005].

HETEROTOPIC BONE ASSAY

The transduced cells were resuspended at a concentration of 5×10^6 cells/100 μl of PBS and then delivered through intramuscular injection into the hind limb quadriceps muscle of C57BL/6 or $TRVP1^{-/-}$ mice (8–12 weeks old; Jackson Laboratories, Bar Harbor, ME). Sample sizes are indicated in figure legends. Animals were euthanized, as indicated in the text, either at daily intervals, or 10 days after injection of the transduced cells. Hind limbs were harvested and either placed in formalin or quick frozen and stored at −80°C. All animal studies performed were in accordance with standards of the Baylor College of Medicine, Department of

Comparative Medicine, after review and approval of the protocol by the Institutional Animal Care and Use Committee (IACUC).

CROMOLYN ADMINISTRATIONS

Intraperitoneal injections of sodium cromoglycate (C0399; Sigma-Aldrich, St. Louis, MO) were administered daily (8 mg/kg/day) for 5 days prior to intramuscular injection of transduced cells, and then continued daily throughout the time course of the heterotopic bone assay. Control animals were given intraperitoneal injections of the vehicle, phosphate buffered saline (PBS), following the same treatment regimen as experimental animals. Animals were euthanized at specified time points following injection of transduced cells.

IMMUNOHISTOCHEMICAL ANALYSIS

Mouse hind limbs were isolated, formalin fixed, and cut in half prior to decalcification, processing, and paraffin embedding. The tissues were serial sectioned (5 μm), and every fifth slide subject to hematoxylin and eosin staining as previously described [Olmsted-Davis et al., 2007]. Serial unstained slides were used for immunohistochemical staining (either single- or double-antibody labeling), using methods outlined previously. For double-antibody labeling, samples were treated with both primary antibodies simultaneously, followed by washing and incubation with respective secondary antibodies, used at 1:500 dilution, to which Alexa Fluor 488, 594, or 647 (Invitrogen, Carlsbad, CA) was conjugated. Primary antibodies were used as follows: Substance P, rat monoclonal, used at 1:250 dilution (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and CGRP, rabbit polyclonal, used at 1:250 dilution (Biomol/Enzo LifeSciences, Plymouth Meeting, PA), neurofilament, mouse monoclonal, (Sigma, St Louis, MO) used at 1:200 dilution, Klf4, goat polyclonal (R&D Systems, Minneapolis, MN) used at 1:200 dilution, nanog, goat polyclonal (Novus Biologicals, Littleton, CO) used at 1:250, osterix, goat or rabbit polyclonal (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) used at 1:200, and VWF, rabbit polyclonal, (Chemicon, Billerica, MA) used at 1:250. Primary antibodies were either diluted in PBS and 10% serum of the species in which the secondary antibody was generated, or for mouse primary antibodies, staining was performed using the M.O.M. Kit (Vector Labs, Burlingame, CA). Tissues were mounted and counterstained using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Stained tissue sections were examined using an Olympus BX41 microscope equipped with a reflected fluorescence system, using a 20×/0.75 NA objective lens 10×, 20×, 40×, 100×.

TOLUIDINE BLUE STAINING

Decalcified, paraffin embedded tissue sections were stained with toluidine blue, to specifically identify mast cells. Toluidine blue O (Sigma, Chemical Company, St Louis, MO) was reconstituted in 70% ethanol, and then diluted at a ratio of 1:10 in 1% sodium chloride (pH = 2.3) for subsequent staining. After deparaffinization and hydration, sections were stained in the working solution of toluidine blue for 10 min, washed, dehydrated, and cover-slipped with resinous mounting medium. The number of toluidine blue (TB) positive cells in an area of the tissue was calculated by adding the number of positive cells counted in each of the fields taken within the area. The area fraction of TB^+ cells was defined as the total number of TB^+ cells within the area divided by the number of fields taken in that area. The TIFF images of the histological sections were first inverted using ADOBE Photoshop. In the inverted images, the mast cells appeared as bright spots. The number of mast cells in each inverted image was counted by segmenting the cells using FARSIGHT (RPI, Troy, New York). The area fraction was measured for five different fields, and the average area fraction was calculated for control and BMP-treated tissues for every fifth slide sectioned throughout the entire hind limb. The area fractions of TB^+ cells in the control and the BMP-treated tissues on day 2 were 3 and 8.33, respectively. Based on the Student's *t* test, the *P* value for the day 2 data was 0.02.

PROTEIN EXTRACTION AND ANALYSIS

Protein from the entire quadriceps muscle, injected with either Ad5BMP2 or Ad5empty transduced cells, was isolated using the Total Protein Extraction Kit (Millipore, Billerica, MA), following manufacturer's instructions. Muscle samples (n = 4) were collected every day, for 6 days following injection. Total protein concentrations of each sample were determined using the BSA Protein Assay Kit (Pierce/ThermoScientific, Rockford, IL). Quantification of protein levels of both Substance P and CGRP were assayed by Enzyme Immunoassay (EIA) (EK-061-05 and EK-015-09; Phoenix Pharmaceuticals, Inc., Burlingame, CA). For each EIA assay, samples were equally loaded based on the total protein concentration, and measured in duplicate. Results from each day were averaged, and the difference in protein levels in control and BMP2 samples assessed by standard *t*-test analysis. The differences in protein levels over time were analyzed by one-way analysis of variance tests (ANOVA) and post-hoc Bonferroni multiple comparisons tests comparing time points, using Stata Ver II (College Station, TX).

MICROCOMPUTED TOMOGRAPHY

Micro-CT exams were obtained of the left and right legs at 15 μm resolution (eXplore Locus SP; GE Healthcare, London, ON, Canada). A hydroxyapatite phantom was scanned alongside each specimen and was used to convert the scan data from arbitrary units to units of equivalent bone density. The scans were thresholded to exclude any tissue with a density less than 100 mg/cc, and the tissue volume within the region of interest was calculated as a measure of the total amount of mineralized tissue. The resulting data were analyzed by one-way analysis of variance to identify differences.

RESULTS

INDUCTION OF SUBSTANCE P AND CGRP THROUGH DELIVERY OF AdBMP2 TRANSDUCED CELLS

To determine if BMP2 directly activates expression of the neuroinflammatory proteins SP and CGRP during heterotopic ossification, proteins were isolated from tissues at daily intervals, starting 24 h after delivery of AdBMP2 or Adempty (control virus) transduced cells, through the appearance of heterotopic bone. Quantification of protein levels of SP and CGRP within the tissues, through ELISA, is shown in Figure 1A and B, respectively. Both proteins appear to be significantly elevated ($P \leq 0.0005$), compared

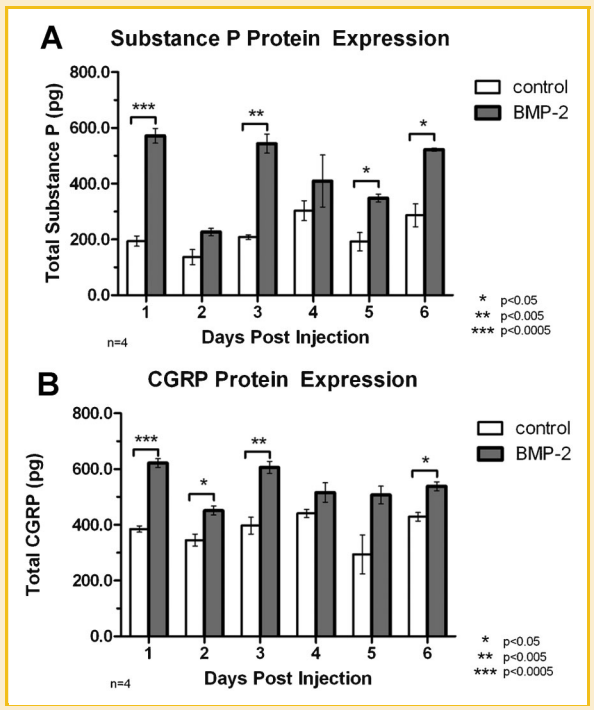

Fig. 1. Quantitation of substance P and CGRP protein by ELISA. Soft tissues, which encompass the site of new bone formation, were isolated at daily intervals from animals receiving either AdBMP2 (BMP2) or Adempty (control) transduced cells, and protein extracts were generated. **A:** Substance P total protein was quantified and statistically significant changes between the groups, as denoted by an asterisk, determined using a standard *t*-test; n = 4. **B:** CGRP total protein was quantified and statistically significant changes between the groups, as denoted by an asterisk, determined using a standard *t*-test; n = 4. * Denotes statistical significance. Error bars represent ±SEM (standard error of the mean).

to controls, within 24 h after induction of HO, and again at 72 h ($P \leq 0.005$) and 6 days ($P \leq 0.05$) after induction. Expression, therefore, appeared somewhat cyclical, and statistical analyses, using a one-way ANOVA with a post-hoc Bonferroni test for comparison between time points, verified a significant drop in SP and CGRP between days 1 and 2 ($P \leq 0.005$). This was followed by a significant rise between days 2 and 3 ($P \leq 0.005$). The data suggests that BMP2 induced a substantial and immediate release of these proteins, which was attenuated, but then continued for the remainder of endochondral bone formation, through the appearance of mineralized bone (Fig. 1).

Tissues were next immunostained for the presence of SP and CGRP and analyzed to determine if the expression of these factors was indeed associated with nerves. Figure 2 shows representative images of the expression of CGRP (red) and SP (green) within the tissues isolated 3 days after receiving either AdBMP2 or Adempty transduced cells. We observed a small amount of positive CGRP (red) and SP (green) expression associated with a mature nerve structure within control tissues, but expression was not found within the muscle itself (Fig. 2F and G). In contrast, in tissues receiving BMP2, CGRP and SP expression was found either within and adjacent to the nerve (CGRP, Fig. 2B) or adjacent to the nerve (SP, Fig. 2C). This suggests that the expression of these factors is associated with BMP2, as predicted [Bucelli et al., 2008].

INHIBITION OF HO IN ANIMALS LACKING TRPV1

The induction of neuroinflammatory mediators occurs through activation of sensory neurons by localized stimulus, or, in this case, secretion of BMP2. To determine if induction of neuroinflammation is contributing to HO, bone formation was quantified in animals that lacked TRPV1 ($TRPV1^{-/-}$), resulting in a functional loss of activity of sensory neurons. These $TRPV1^{-/-}$ animals lack a functional

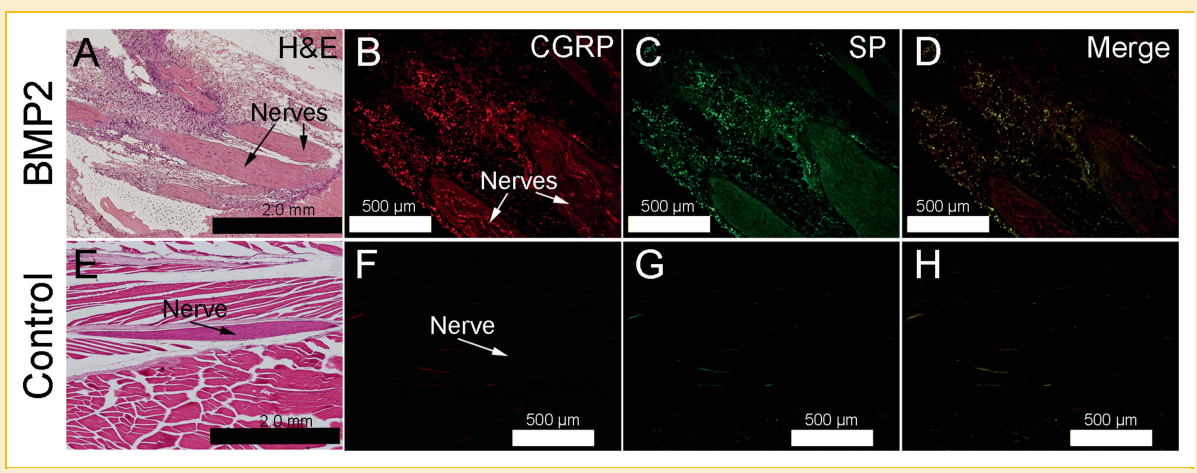

Fig. 2. Photomicrographs of substance P and CGRP protein expression in tissues isolated 3 days after induction of HO. Tissues receiving cells transduced with AdBMP2 (BMP2) or Adempty cassette (control) were isolated 3 days after induction and immunostained with antibodies against substance P and CGRP. Expression of these factors was found within and adjacent to the nerves in tissues receiving BMP2, but was minimal in tissues receiving the Adempty cassette transduced cells. Hematoxylin and eosin stained serial sections, adjacent to the section used for immunostaining, 3 days after receiving AdBMP2 transduced cells (**A**) or Adempty cassette transduced cells (**E**). Arrows mark the nerves. Positive staining by the nerves in tissues receiving BMP2 (**B** and **C**) or by the nerves in control tissues (**F** and **G**) for CGRP (**B** and **F**; red color) or substance P (**C** and **G**; green color). **D:** A merged image of (**B**) and (**C**). **H:** A merged image of (**F**) and (**G**).

cationic channel on peripheral, sensory nerve terminals, which regulate neurogenic inflammation [Patapoutian et al., 2009]. We quantified the changes in SP and CGRP protein expression within tissues isolated from these knockout animals, and observed a significant suppression compared to the wild type counterpart (Supplemental Fig. S1), although we did observe a slight increase in their expression upon delivery of BMP2.

HO was induced in both TRPV1$^{-/-}$ and wild type mice ($n = 7$), and, after 10 days, the resultant bone formation was quantified through micro-computed tomography (μCT). Figure 3A shows a representative three dimensional reconstruction of the bone formation. Heterotopic bone volume within TRPV1$^{-/-}$ mice was inhibited significantly ($P \leq 0.05$), as compared to wild type mice (Fig. 3B).

NEUROINFLAMMATORY ASSOCIATED CHANGES IN MAST CELLS

The reduction of HO when there is a lack of functional TRPV1 signaling suggests that this pathway may be functionally important to the process of HO. The next step in neuroinflammatory signaling involves recruitment of mast cells and their resultant degranulation, for the release of key enzymes involved in processing proteins essential for inflammatory signaling and recruitment. To determine whether mast cells were recruited to the site of new bone formation, muscle tissues from the hind limbs of wild type mice injected with AdBMP2 or Adempty transduced cells were isolated at daily intervals and then serially sectioned in entirety for quantification.

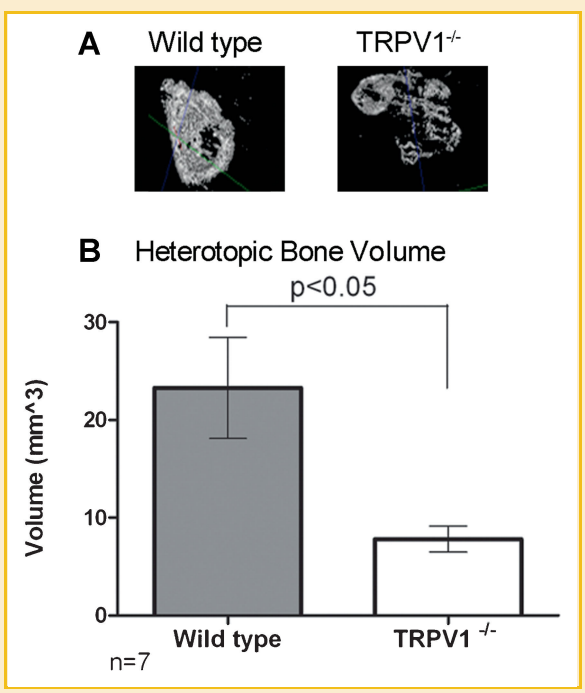

Fig. 3. Microcomputational analysis of heterotopic ossification 10 days after induction with AdBMP2 transduced cells, in C57/BL6, wild type or TRVP1$^{-/-}$ mice. **A:** Three-dimensional reconstructions of representative samples for each group. **B:** Quantitation of bone volume. Statistically significant changes between the groups was determined using a one-way analysis of variance; $n = 7$; $P < 0.05$. Error bars represent $\pm$SEM.

There appears to be a trend toward more mast cells within the tissues undergoing HO, as compared to the control tissues (Fig. 4A). However, only day 2 shows a statistically significant increase in the number of mast cells.

Since mast cells are known to migrate throughout the tissues, co-localization with specific tissue structures was also noted. As seen in Figure 4C, mast cells appeared to be scattered throughout the control tissues. However, within the tissues receiving AdBMP2 transduced cells, mast cells associated only with the nerves (Fig. 4B), in tissues isolated 2 days after induction of bone formation. As bone formation continues, the mast cells within the tissues receiving BMP2 continue to be localized within the nerve itself; however, a subset also appear within the vessel structures (data not shown). We did not see mast cells localizing within the nerve structures in control tissues at any time point.

Mast cell degranulation leads to the release of degradative enzymes, such as tryptase and chymase. These enzymes are known to degrade or process other proteins, leading to their activation. Many of the enzymes are involved in tissue remodeling, including the nerve structure itself. To determine if mast cell degranulation could be a factor in heterotopic ossification, animals were pretreated with the drug sodium cromoglycate (cromolyn), which has been shown to prevent mast cell degranulation [Cox, 1967]. Following the pre-treatment with either cromolyn or a vehicle control (PBS), HO was induced and the resultant bone formation quantified 10 days later. Figure 5A shows representative images of three dimensional reconstructions of the resultant HO formation after cromolyn or vehicle control, PBS treatment. As can be seen in Figure 5B, quantification of bone volume of the HO shows a significant ($P \leq 0.05$) decrease in animals after cromolyn treatment.

The data collectively suggest a molecular model in which sensory neurons signal to induce neuro-inflammatory mediator expression and mast cell migration and degranulation, which ultimately facilitate HO. Since others have shown that progenitors reside within the nerve sheath [Adameyko et al., 2009] and can expand upon nerve remodeling after injury, we analyzed the nerves isolated in hind limb tissues from cromolyn or vehicle (PBS) treated animals after induction of HO by delivery of AdBMP2 transduced cells. We hypothesized cromolyn treatment would block nerve remodeling, and thus, the ultimate release of progenitors perhaps residing within the nerve. The tissues were immunostained for expression of a variety of stem cell markers, and, intriguingly, we observed changes in the subset of markers related to pluripotency. Figure 6A–F shows representative photomicrographs of tissues immunostained for expression of Nanog and Krüppel-like family of transcription factor 4 (Klf4) within the tissues isolated 2 days after induction of HO. As seen in Figure 6, in animals that received cromolyn, cells positive for these factors were observed throughout the nerve (Fig. 6B and C), as demonstrated by neurofilament (NF) staining. However, in tissues isolated from animals that received vehicle alone, these markers were significantly reduced, to completely absent, throughout the nerve (Fig. 6E and F), when compared to animals receiving cromolyn. We observed nanog^{+} and Klf4^{+} cells within tissues isolated from animals injected with BMP2 producing cells without cromolyn treatment, but such cells were rare and often did not co-localize with the nerve. In addition, control tissues from animals

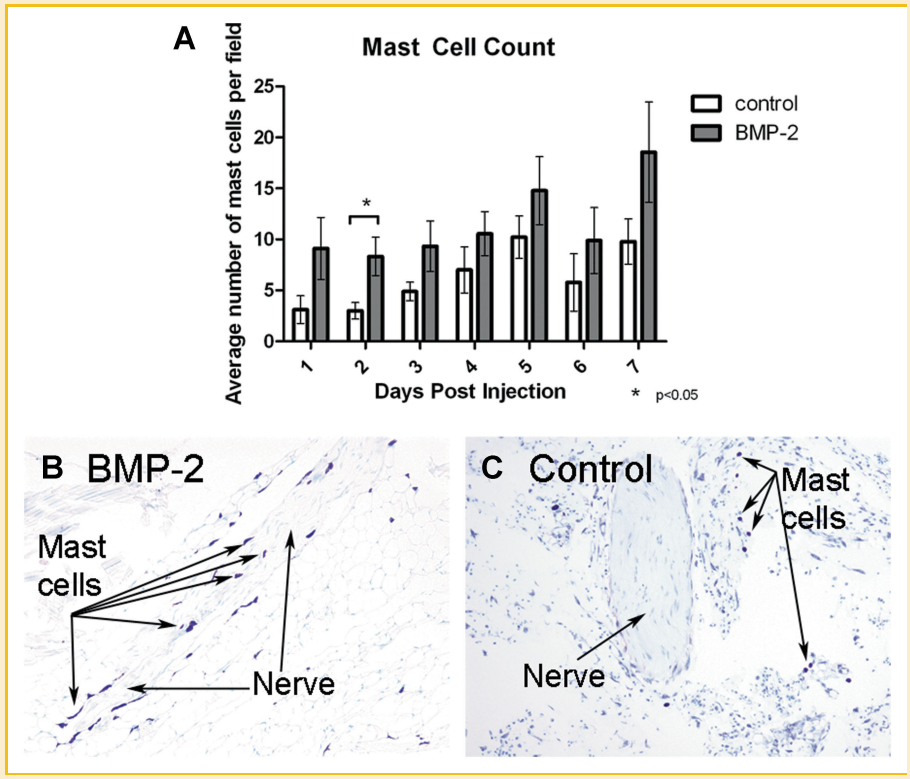

Fig. 4. Quantitation of mast cells in tissues surrounding the area of new bone formation. Tissue sections were stained with the mast cell stain, toluidine blue. Photomicrographs of random fields (five per section) were taken at 10× magnification, for every fifth slide, throughout the entire hindlimb. Each field equals 5.2 mm^2. **A:** Quantitation of the average number of mast cells within the tissues at daily intervals after induction of HO. Statistically significant changes were determined using a standard *t*-test; n = 3 biological replicates. Seven slides were analyzed per tissue, and five fields per slide (35 images quantified per sample/time point). * Denotes statistical significance. Error bars represent ±SEM. **B:** Representative photomicrographs of tissues isolated 2 days after receiving AdBMP2 (BMP2) or Adempty (control) transduced cells, stained with toluidine blue. Positive cells are highlighted with arrows.

receiving Adempty transduced cells, which were either cromolyn or vehicle treated, did not express these stem cell markers in the nerve (Fig. 6G–L), indicating that the observed changes in marker expression can be attributed to an effect of both cromolyn and BMP2. Both nanog and Klf4 have been implicated in maintenance of the pluripotential phenotype observed in embryonic stem (ES) cells [Hanna et al., 2009], with Klf4 actually enhancing the expression of the nanog gene in ES cells [Nakatake et al., 2006; Jiang et al., 2008]. Interestingly, the osteoblast specific transcription factor osterix was also found associated with the nerve sheath (Fig. 7). Osterix expression was observed in the nerve by co-staining with the neural marker neurofilament (Fig. 7G, H, and I), in tissues isolated 2 days after BMP2 induction, in the presence of cromolyn. These osterix positive cells also appeared to co-localize with a portion of the primitive stem cell factors, suggesting that a subset of the cells may be undergoing differentiation (Fig. 7A–F).

DISCUSSION

Heterotopic ossification is a disorder involving rapid bone formation within muscle, tendon, and ligaments, adjacent to skeletal bone, and it has been linked to an elevation in BMP2 signaling [Shore and Kaplan, 2010]. Further, the incidence of HO appears to be dramatically increased in individuals who have sustained traumatic injury to the nervous system [Forsberg et al., 2009]. Here we determined whether localized changes in BMP signaling, which lead to heterotopic bone formation, can also alter peripheral nerve signaling through induction of neuroinflammation. Our results suggest that in the presence of BMP2, sensory neurons express mediators of neuroinflammation, resulting in the recruitment of mast cells and remodeling of the nerve structure.

BMP2 has been shown previously to induce the expression of the neuroinflammatory mediators, substance P and CGRP, in sensory neuron cultures [Bucelli et al., 2008]. Here we quantified changes in these mediators, in vivo, after delivery of cells expressing BMP2. We found a significant and immediate elevation of both proteins, in relation to the control, which received the same cells transduced with an Adempty virus. Interestingly, we observed a strong correlation in elevation of these mediators, immediately following our delivery of BMP2. However, as the process continued over time, we observed a cyclical pattern in the expression of these mediators, with a significant decline in expression on day 2, followed by a significant rise in expression on day 3, and a trend towards another increase in expression by day 6. Although BMP2 would presumably be expressed for the first 3–4 days, prior to the rapid clearance of the

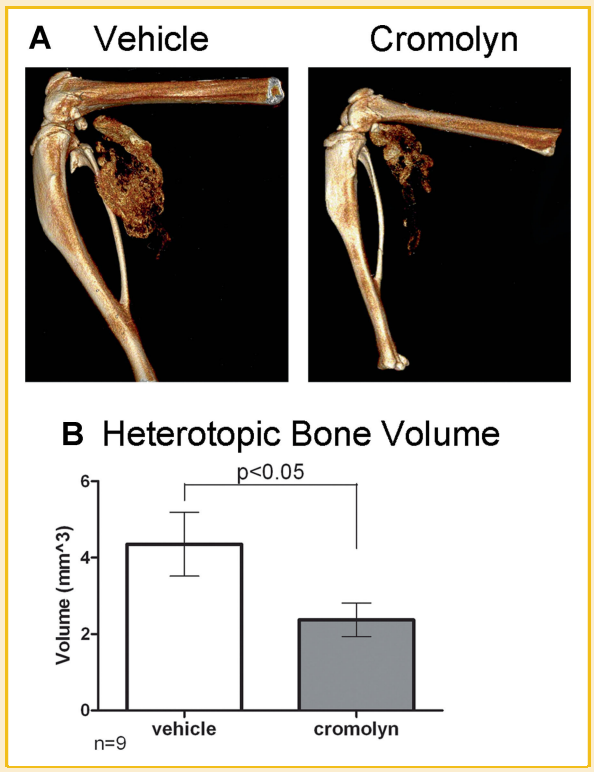

Fig. 5. Microcomputational analysis of heterotopic ossification 10 days after induction with AdBMP2 transduced cells, in mice pre-treated with either cromolyn or vehicle control (PBS). **A:** Three-dimensional reconstructions of representative samples for each group. **B:** Quantitation of bone volume. Statistically significant changes between the groups was determined using a one-way analysis of variance; $n = 9$; $P < 0.05$. Error bars represent ±SEM.

cells [Fouletier-Dilling et al., 2007], the kinetics of BMP2 receptor signaling in this model is unclear. Intriguingly, one of the first steps is the rapid formation of brown adipocytes within the tissues [Olmsted-Davis et al., 2007]. We have previously shown brown adipocytes to be necessary for patterning of the new bone, by their unique capacity to regulate the oxygen microenvironment, not only by stimulation of new vessels, but also by uncoupling of aerobic respiration and "burning" of oxygen [Olmsted-Davis et al., 2007]. The result of this uncoupling is a release of energy as heat, which could potentially re-stimulate sensory neurons to respond and release substance P and CGRP. This could potentially explain the observed cyclical nature of the response, suggesting secondary or tertiary signaling events.

Performing the assay in animals lacking TRPV1, we saw a significant decrease in the volume of heterotopic bone formed, compared to animals with functional TRPV1. The suppression, rather than complete ablation, suggests that other TRPV family members present on sensory neurons may also contribute to the induction of HO. Although we do not rule out alterations in other peripheral nerve signaling to the central nervous system in these animals, both substance P and CGRP were found to be significantly decreased in the TRPV1$^{-/-}$ mice. We still observed a trend towards an increase in substance P and CGRP upon addition of the AdBMP2 transduced cells. However, this was not above the normal background levels observed in wild type mice, nor was it statistically significant for CGRP, and the induction was over three folds lower for SP, so it is unclear whether this contributes to HO. The result that BMP2 does not induce SP or CGRP in TRPV1$^{-/-}$ mice is not surprising, as it has been previously shown that TRPV1 induces SP in response to capsaicin [Theriault et al., 1979] and that TRPV1 also controls heat- and acid-induced CGRP release from sensory nerves [Kichko and Reeh, 2009]. In addition, previous studies have revealed decreased injury-induced neuropeptide release in TRPV1$^{-/-}$ mice [Wang and Wang, 2005]. While TRPV1 is unquestionably involved in pain and neuroinflammation, TRPV has also been found to be involved in diabetes [Razavi et al., 2006] and obesity [Motter and Ahern, 2008]. Whether this is by the same mechanism proposed here, or by alternative mechanisms, remains undetermined. Consequently, deletion of TRPV1 could have additional pleiotropic effects. TRPV1$^{-/-}$ mice receiving Adempty transduced cells did not produce heterotopic bone, which is in line with our previous findings that Adempty transduced cells have not produced HO in any animal model we have tested [Olmsted-Davis et al., 2002].

Mast cells are known to be recruited to nerves during times of neuroinflammation. Upon degranulation, mast cells release a number of digestive factors, chymases, tryptases, and other enzymes, which can cleave proproteins, leading to their activation. These factors appear to be essential for tissue remodeling of not only the nerve, but also other surrounding tissues, including the vasculature [Johnson et al., 1988; Richardson and Vasko, 2002; Kleij and Bienenstock, 2005; Schaible et al., 2005; Kulka et al., 2008]. Nerve remodeling is thought to be part of neurite outgrowth, or the ability to remodel and extend neurons. Perhaps this process is utilized to innervate the newly forming HO. Alternatively, Adameyko et al. [2009] recently demonstrated the presence of a stem cell population residing within peripheral nerves that would migrate from the nerve to undergo melanocyte differentiation. We quantified the number of mast cells after induction of HO and found a significant elevation in this population within 48 h, when compared to tissues receiving the control cells. We observed an upward trend in the number of mast cells on all days. However, perhaps due to the immune response evoked to clear the injected cells, there was also an increase in mast cell numbers in the control tissues, leading to a significant difference only on day 2. Further, we observed the mast cells, within the first 48 h, associating with the nerves and within the nerves, as compared to control tissues where the mast cells were usually located randomly throughout the tissues. It is intriguing that we observed the most significant difference at these early stages, since this appears to parallel our findings for the release of SP and CGRP within the tissues, suggesting mast cells may be recruited after release of these factors.

Although not shown here, we did observe the mast cells associating with vessels, as well as nerves, later in the time course, suggesting that there is also a secondary vascular remodeling that may be triggered through this process. In line with these results, mast cells have been observed in the human, genetic disease of HO, fibrodyplasia ossificans progressiva [Gannon et al., 2001]. Also consistent with our observations, mast cells have previously been shown to be recruited by SP and CGRP [Yano et al., 1989; Itoh et al.,

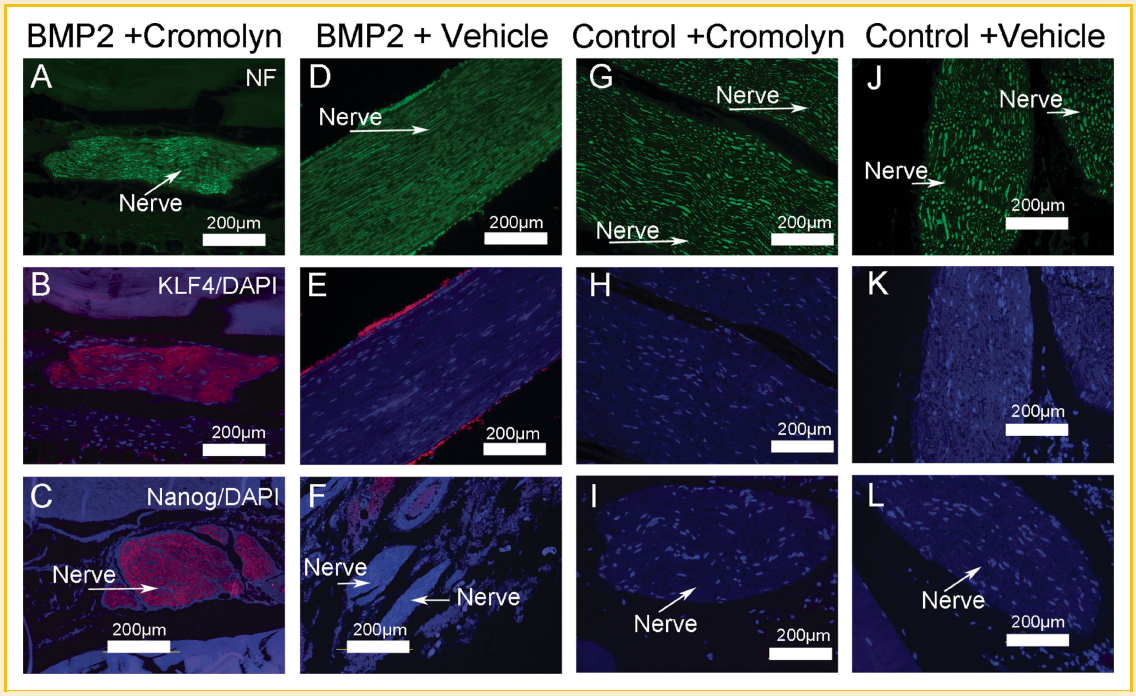

Fig. 6. Peripheral nerves contain primitive stem cell markers after BMP2 induction in the presence of cromolyn. Photomicrographs of tissues isolated 2 days after induction of HO. Tissues from animals receiving cells transduced with AdBMP2 (BMP2) and pre-treated with either cromolyn (**A–C**) or vehicle, PBS (**D–F**) were isolated 2 days after induction, and immunostained with antibodies against stem cell markers, Klf4 and Nanog. Expression of these factors was found to co-localize to nerves residing within the area of new bone formation, upon cromolyn treatment. Tissues from animals receiving cells transduced with Adempty cassette (control) and pre-treated with either cromolyn (**G–I**) or vehicle (**J–L**) were isolated and immunostained the same. Nerves were identified by neurofilament (NF) staining (**A, D, G, J**; green color). Positive staining for Klf4 (**B**; red color) or Nanog (**C**; red color) was observed throughout the nerves in tissues isolated from animals receiving cromolyn at 2 days after BMP2 induction. In comparison, the nerves in BMP2 treated animals receiving vehicle appeared to lack positive staining for either Klf 4 (**E**; red color) or Nanog (**F**; red color). The nerves in animals receiving Adempty transduced cells also appeared to lack positive staining for either Klf4 (**H, K**) or Nanog (**I, L**), under both conditions. Tissues were counterstained with DAPI (**B, C, E, F, H, I, K, L**; blue color), which stains the nucleus of cells.

2010] and indeed have multiple receptors that bind to these inflammatory mediators [Kulka et al., 2008]. However, other modulators can bind and recruit mast cells, including neurokinin A, derived from pre-protachykinin 1, which is also the precursor for SP [Itoh et al., 2010].

Additionally, mast cells, like platelets, store serotonin, presumably derived from the gut, and release it in an active form upon degranulation [Theoharides et al., 1982]. Serotonin, which has recently been linked to skeletal bone remodeling [Ducy and Karsenty, 2010], is then capable of stimulating sympathetic neurons, which can ultimately regulate adipose production. Serotonin released by the gut has been reported to favor bone resorption during bone remodeling, whereas serotonin released in the hypothalamus appears to enhance bone formation and increase osteogenesis. Although it is still unclear how serotonin release contributes to HO, we have previously reported that brown adipocytes are made rapidly in the local tissues, presumably through activation of sympathetic neurons [Salisbury et al., 2010]. Thus, it seems likely that serotonin release may aid in the production and activation of adipocytes necessary for creating a microenvironment conducive for formation and patterning of bone.

We next looked at whether the nerve remodeling was releasing cells that were essential to bone formation. As noted above, it has been previously demonstrated that precursors in peripheral nerves are the origin of skin melanoctyes. Therefore, mast cell degranulation, and subsequent nerve remodeling, was blocked using cromolyn, and we observed a significant decrease in HO. We next analyzed the nerves from these animals and found an increase in cells expressing markers of early stem cells (nanog and Klf4). These primitive markers were sporadic in the nerves of untreated animals, but completely covered the nerve in the cromolyn treated animals. This not only suggests that the early tissue changes lead to expansion of these cells, but also that the pool size of these cells within the nerve of untreated animals is extremely low, due to concomitant and rapid migration and differentiation. However, blockade of these latter steps with cromolyn leads to accumulation of these cells expressing primitive markers within the nerve. We note here, however, that the mechanisms of cromolyn action are incompletely understood. Although cromolyn is widely characterized as a "mast cell-stabilizer" (i.e., an agent that blocks the release of mast cell mediators following appropriate activation of the cell) that can suppress mouse mast cell function in vivo, its molecular targets are neither fully defined nor restricted to mast cells [Galli et al., 2008]. Moreover, while the mechanism of action of cromolyn mainly involves mast cell degranulation [Cox, 1967], other mechanisms, such as inhibition of neutrophils and eosinophil

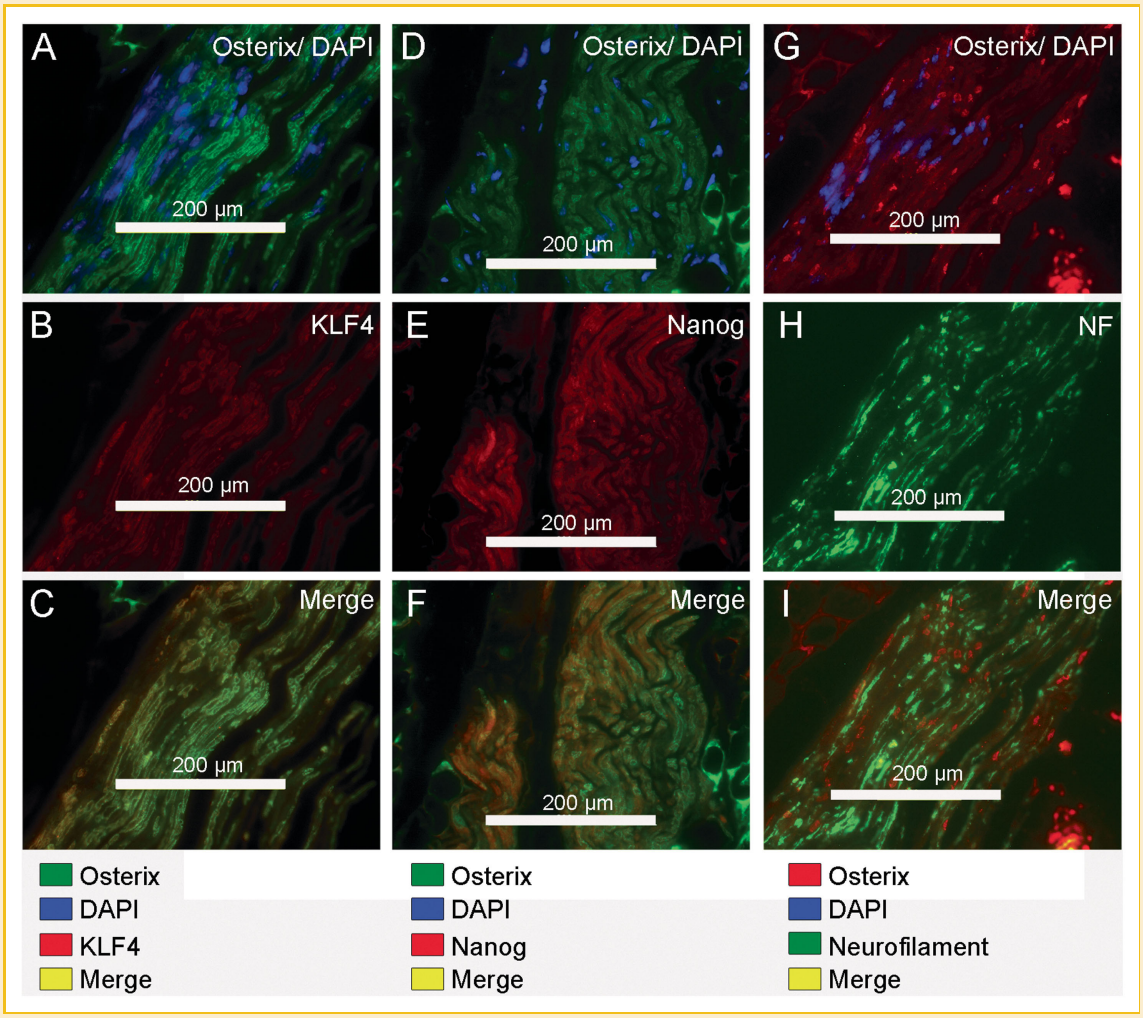

Fig. 7. Peripheral nerves contain osterix-expressing cells after BMP2 induction in the presence of cromolyn. Photomicrographs of osterix expression within tissues isolated 2 days after BMP2 induction in the presence of cromolyn. Images of osterix expression (**G**; osterix red color, DAPI blue color) associated with the nerve (**H**; neurofilament, NF, green color). **I:** Osterix and NF, is a merger of (**G**) and (**H**). Images of the expression of osterix (**A**, **D**; osterix green color, DAPI blue color) and Klf4 (**B**; red) or nanog (**E**; red). **C:** Osterix and Klf4, is a merger of (**A**) and (**B**). **F:** Osterix and nanog, is a merger of (**D**) and (**E**).

induced chemotaxis [Bruijnzeel et al., 1990], have also been described.

To our surprise, we observed osterix positive cells on the nerve as early as day 2, in the presence of cromolyn. There were also cells that expressed primitive stem cell factors, which appeared to simultaneously express osterix, suggesting that these cells are osteoblast precursors. The majority of osterix positive expression was associated with the nerve. We also observed Klf-4^{+} and nanog^{+} cells that were not associated with osterix, suggesting that these cells may have other potentials. Besides osteoblasts, another possible fate of these cells may be brown adipocytes, which we have shown previously to be critical for reduction of the oxygen tension in the microenvironment for cartilage formation [Olmsted-Davis et al., 2007] and for secreting VEGF for vessel formation [Dilling et al., 2010]. It has recently been noted that the *Misty* mouse phenotype [Sviderskaya et al., 1998], which is deficient in brown fat, is caused by a mutation in dock 7 [Blasius et al., 2009], a neuronal factor that regulates Schwann cell migration and neuronal polarity. It is intriguing to speculate that brown fat progenitors may also reside in peripheral nerves, particularly since TRPV1 responds to heat [Szallasi et al., 2007]. Additionally, it is interesting that the mutation in a single neuronal protein, dock7, not only dramatically increases HO in the *Misty* mouse [Olmsted-Davis et al., 2007], but also causes severe osteoporosis in the skeletal bone (Rosen C., unpublished). Further, we previously demonstrated the rapid formation of new vessels early after BMP2 induction [Dilling et al., 2010], suggesting that several types of tissues are being assembled simultaneously during this period. Osterix has previously been suggested to play a role in osteoblast lineage commitment of progenitors, suppressing the adipose phenotype [Cheng et al., 2003]. Perhaps the early osterix expression, 4 days prior to the appearance of osteoid matrix, may be part of a regulatory mechanism to preserve these cells for future osteogenic fate. Finally, although not highlighted in this manuscript, we did observe osterix positive cells,

at later times, in vessel-like structures that co-aligned with early endothelial markers, such as flk 1, which we have previously identified as characteristic of this early vasculogenesis [Dilling et al., 2010]. This notion supports the work of Lounev et al. [2009], suggesting that osteoblast progenitors reside within the newly forming vessels, have a Tie 2 marker, and are not derived from marrow [Kaplan et al., 2007]. Other investigators have also provided evidence for this concept of osteoblast progenitors being associated with the vasculature [Kolf et al., 2007; Medici et al., 2010].

This study is the first step in identifying a potential direct role for the peripheral nervous system in the induction of heterotopic ossification. The data suggest that early neuroinflammation, elicited in the presence of BMP2, may be capable of expanding a population of cells within the nerve, which can migrate and potentially contribute to a number of structures, rapidly assembling to produce HO. Suppression of these steps significantly decreases HO formation. Although it is unclear what affects this may have on the adjacent skeletal bone, the data suggest that there is direct communication with the hypothalamus, which could, in part, signal to impact bone remodeling. Understanding these earliest steps of HO will, for the first time, provide us novel targets for therapeutic intervention, which may ultimately lead to effective treatments. Finally, it is conceivable that such a mechanism could play a role in many other disease states, including neurofibromatosis and vascular calcification.

ACKNOWLEDGMENTS

This work was supported by grants from the US Department of the Army, Orthopedic Trauma to ARD (DAMD W81XWH-07-1-0214) and EAOD (DAMD W81XWH-07-1-0215), as well as a grant from NBIB (EB005173) and from the NIH (T-32 HL092332-06) to ER, and a Pre-doctoral fellowship from the American Heart Association (AHA 10815339F) and Kirschstein-NRSA (T32 HL092332-08) to ES. The authors thank Hua Chen of the Vector Development Laboratory, Baylor College of Medicine for vector preparation.

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