Progenitors in Peripheral Nerves Launch Heterotopic Ossification

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Key Words. Heterotopic ossification • Bone morphogenetic type 2 • Neural stem cells • Blood-nerve barrier • Trauma

ABSTRACT

Studies presented here, using a murine model of bone morphogenetic protein type 2 (BMP2)-induced heterotopic ossification (HO) show that the protein initiates HO by signaling through progenitors in the endoneurium of peripheral nerves. In the mouse, these cells were identified in the endoneurium one day after BMP2 induction using antibody against phosphoSMAD (PS) 1, 5, and 8. Studies conducted in a tracking mouse that contains a tamoxifen-regulated Wnt1-Cre recombinase crossed with a td Tomato red (TR) reporter (Wnt1CreERt:Ai9Tm) confirmed their neural origin. In this model both BMP2 induction and tamoxifen are absolutely required to induce TR. SP7 (osterix1)TR+ cells were found in the endoneurium on day 1 and associated with bone on day 7. Quantification of TR− and TR+ cells isolated by fluorescence-activated cell sorting showed that all SP7+ cells were found in the TR− population, whereas about 80% of the TR+ cells expressed SP7. Pre-chondrocytes (Sox 9+) and transient brown fat (tBAT, UCP1+) also coexpressed TR, suggesting that the progenitor in nerves is multi-potential. The endoneurium of human nerves near the site of HO contained many PS+ cells, and SP7+ cells were found in nerves and on bone in tissue from patients with HO. Control tissues and nerves did not contain these PS+ and SP7+ cells. Some osteoblasts on bone from patients with HO were positive for PS, suggesting the continued presence of BMP during bone formation. The data suggests that the progenitors for HO are derived from the endoneurium in both the mouse model of HO and in humans with HO.

SIGNIFICANCE STATEMENT

Heterotopic ossification is bone formation at nonskeletal sites. It occurs in traumatic injury and thus far there is no treatment. This article describes new research on the mechanism of this disease, and indicates that most or all of the progenitor cells arise from cells within peripheral nerves. Evidence is also presented that such progenitors are multipotent in that they become not only osteoblasts, but also chondrocytes and transient brown adipocytes. The disease process is therefore quite unique and may lend itself to novel and specific therapies.

INTRODUCTION

Heterotopic ossification (HO), the formation of bone in the muscle, other soft tissue, or any non-skeletal site can cause severe pain and disability. It often requires the patient to undergo additional surgery. A particularly frustrating problem in amputees is the growth of bone within the amputation stump, making prosthesis wear difficult or impossible. Such heterotopic bone also develops spontaneously near the joints in many patients with severe burns, an injured spinal cord, and traumatic brain injury [1,2]. Tentative inhibitors, such as low dose radiation that have some efficacy in preventing HO in patients at high risk, cannot be implemented in the majority of cases. Thus, there are currently no available efficacious treatments. Although the incidence of HO in the general population is fairly low, approximately 11% of all musculoskeletal injuries, it is a significant problem within the military where the incidence is approximately 60%–70% of all traumatic extremity injuries involving amputations [3].

Previous studies to determine the location and cellular phenotype of the osteoprogenitors in heterotopic ossification (HO) have been controversial and have specified that the progenitor is a vascular endothelial cell [4], a multipotent progenitor resident in the skeletal muscle interstitium [5,6], or a neural cell [7]. Although these reports do not agree on its origin, surprisingly they agree on several markers associated with the osteoprogenitor phenotype. These markers include PDGFRA, Tie-2, and SP7 [4, 5, 7]. Even studies of
human osteoprogenitors involved in HO induced by traumatic injury, although passaged in vitro, confirm the presence of these markers on the cells [8–10]. Many reports suggest that the variation in origin could be due to the variation in HO models.

Studies using a model where HO is induced through sustained cellular release of physiological levels of BMP2, through delivery of adenovirus-transduced cells, provides a reproducible method to study this de novo bone formation [11]. One of the earliest steps in the process is the remodeling of the epineurial or outer matrix structure of the peripheral nerves [7,12,13] near the site of HO and associated neurogenic inflammation [12,14]. This process involves mast cells, which degranulate and contribute to the activation of the sympathetic nervous system [12] ultimately leading to the formation of brown adipocytes from cells derived from the perineurial layer of the peripheral nerves [13]. These cells express the neural migration protein HNK1 and appear to move toward the BMP2 [13].

In addition to accessory cells migrating from the perineurial layer, cells within the endoneurium or axon compartment were found to express the osteoblast-specific factors SP7 and Dlx5 [12, 13]. These SP7+ Dlx5+ cells exit the nerve through the endoneurial vessels and are deposited at the site of new bone formation [7]. The endoneurial osteoprogenitors express the tight junction molecule claudin 5 when in circulation or at the site of new bone formation despite the fact that they do not initially express this marker in the nerve [7]. The observation suggests that these endoneurial cells are responding to the BMP2 and exiting the nerve through the blood-nerve barrier by upregulating the neurovascular tight junction protein claudin 5 [7].

The studies described in this article use an antibody that recognizes cells responding to BMP2 and reveal few if any cells respond initially, all in the neural endoneurium. To confirm that the SP7+ cells from the endoneurium are actually exiting the nerve, enter the circulation, and being deposited at the site of new bone formation, a tamoxifen-regulated td Tomato red lineage-tracking mouse (Wnt1CreErt:Ai9Tm) was used so that cells originating in the endoneurium could be tracked. The results presented here not only confirm the neural origin of the osteoblasts, but also show reporter expression in cartilage and brown adipocytes suggesting that the neural progenitor may be multipotential. To determine if these findings apply to human HO, tissues from patients with early HO were obtained and immunostained. Surprisingly, the patient tissues showed a significant number of cells within the endoneurium responding to BMP, many more than in the mouse model. The data suggests that the endoneurium plays a key functional role in heterotopic bone formation in both mice and humans.

**Materials and Methods**

**Viruses and Cells**

Recombinant adenovirus type 5 capable of BMP2 expression was used as previously described [11] to transfect [15] mouse skin fibroblasts at a multiplicity of infection of approximately 2,500–5,000 viral particle per cell. The purified virus was always less than 100 particle/PUF and was free of replication competent adenovirus.

**Tracking Mice**

All experiments were conducted under an Institutional Animal Use and Care Committee approved protocol in accordance with Organization of Laboratory Animal Welfare. All animals were housed in an AAALAC accredited vivarium under standard conditions in accordance with the Organization of Laboratory Animal Welfare. Mice were randomly selected based on age and health and placed in an experimental group. Each animal was given an experimental number that is linked only to its group in the medical record. Therefore, experimenters involved in data collection and analysis were blinded, and the animal numbers only linked back to groups, for the final data analysis. Group sizes were based on historical power-analysis data; however, all power analysis was repeated after data collection to confirm group sizes were adequate. In these experiments, male C57BL/6 (Jackson Laboratory, Bar Harbor, ME, www.jax.org/) or Wnt1CreErt:Ai9Tm (see below) mice at 6–8 weeks of age were used with the group sizes as indicated.

The Ert Wnt1 Cre transgenic mouse was obtained from Jackson Laboratory (Stock No. 008851). These mice were crossed with the R26R td Tomato red mouse (Stock No. 007914, Jackson Laboratory), which contains a constitutive promoter driving the expression of the fluorescent reporter td Tomato red [16] but with this transgene preceded by a large floxed intervening sequence to form the tracking transgenic mice used in these studies.

The resultant mouse is referred to throughout the manuscript as Wnt1CreErt:Ai9Tm. Tamoxifen (1 mg per mouse, Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com/united-states, T5648) or vehicle control (9:1 (vol/vol), sunflower oil:100% ethanol) was delivered to the mice through subcutaneous injection daily starting 3 days prior to the induction of HO and was given each day including the day of injection of BMP2-producing cells after which tamoxifen injection was stopped. In order to track neural crest stem cells labeled during development, the Wnt1Cre2 (Stock No. 022137) transgenic mouse [17] was purchased from Jackson Laboratories and crossed with the R26R td Tomato red mouse to generate the mouse termed Wnt1Cre2:Ai9Tm. Heterotopic bone formation was established in the mice through an intramuscular injection into the quadriceps of C57BL/6 mouse fibroblasts (1 × 10^5, 5 × 10^5, 1 × 10^6, or 5 × 10^6 in 300 μl of phosphate-buffered saline) transduced with an E1-E3 deleted adenovirus expressing BMP2 [11].

**Immunohistochemistry**

Hind limbs were harvested and the skeletal bone removed so that frozen sections could be prepared, since TR (td Tomato red) does not survive decalcification. Immunohistochemistry encompassing the ectopic bone site was carried out after fixing the tissues with 4% paraformaldehyde, progressing the samples through a gradient of 10%–30% sucrose (wt/vol) in phosphate buffered saline, embedding them in OCT, and cutting 5 μm sections in a cryostat. Reaction of the section with primary antibodies against either mouse SP7 (osterix, Abcam, Cambridge MA, www.abcam.com; 1:200), Dlx5 (Santa Cruz, Santa Cruz, CA, www.scbt.com; 1:200), BGLAP (osteocalcin, Abcam, Cambridge, MA; 1:500), UCP1 (Merck Millipore, Billerica, MA, www.merckmillipore.com; 1:200), Sox9 (R and D Systems, Minneapolis, MN; 1:200), Neurofilament (NF) (Sigma-Aldrich, St Louis MO (1:1000), or NGFR [7] (Abcam, Cambridge, MA; 1:200) was followed by reaction with a secondary antibody conjugated to Alexa fluor 488 so that cells positive for these antigens, but either positive or negative for td Tomato red could be visualized by fluorescent microscopy or fluorescence-activated cell sorting (FACS). According to the manufacturer, the SP7 antibody (Abcam ab22552) has been successfully used in 42 different publications. It reacts with both human and mouse osterix.
Antibody against mouse phosphoSMAD 1, 5, and 8 (PS) was obtained from Cell Signaling Technologies (CST, Danver, MA) and was pretested by CST for its ability to detect BMP2 activation. The reagent recognizes Smad1 and Smad5 protein when phosphorylated at Ser463/465 and Smad 8 protein when phosphorylated at Ser463/465.

Fluorescence-Activated Cell Sorting

Cells were isolated from the muscle with collagenase digestion as previously described [13] before being subjected to FACS using a FACS Aria II cell sorter (BD Biosciences, San Jose, CA) equipped with analyzing software (BD FACSDiva software version 6.1, BD Biosciences). In some instances, these sorted populations were deposited onto glass microscope slides by cytocentrifugation. Either 1 × 10^5, 5 × 10^5, 1 × 10^6, or 5 × 10^6 BMP2-producing cells were injected into the C57BL/6 mouse quadriceps (n = 4 per dose). One day later the mice were euthanized and the limbs injected were frozen and serial sections prepared. These were stained with antibody to mouse PSs 1, 5, and 9 as well as an antibody to mouse NF. This was followed by reaction with secondary antibodies such that the PS was green and the NF red. The number of green cells per 10X field were counted. The average PS cell counts are presented versus dose of cells. One standard deviation unit above and below the average is shown. (p values: 1 × 10^5 to 5 × 10^5 cells, 0.04; 5 × 10^5 to 1 × 10^6 cells, 0.002; 1 × 10^6 to 5 × 10^6 cells, 0.007. (B): Single representative images of 10X fields of the experiment presented in panel (A) from three separate mice. (C): PS and TR are coexpressed in endoneurial cells. Wnt1CreErt: Ai9Tm mice were treated with tamoxifen on days −2, −1, and 0 and each injected with 5 × 10^5 BMP2-producing cells. Frozen sections were prepared and stained with anti-PS antibody followed by a detector antibody conjugated to Alexa fluor 488 (green), DAPI stain (blue). (D): PS is not expressed in the nerves of C57BL/6 mice that were not injected: (Da), NF; (Db), PS. C57BL/6 mice or that were injected with 5 × 10^5 cells transduced with AdEmpty: (Dc), NF; (Dd), PS. Quantification of the fluorescent photomicrographs in Figure 1 show that 95% – 9% of the PS⁺ cells express TR. Abbreviations: BMP2, bone morphogenetic protein type 2; NF, neurofilament; PS, phosphoSMAD; TR, Tomato red.

Figure 1. The target for BMP2 in heterotopic ossification is an endoneurial cell. (A): The number of PS⁺ cells varies with the input of BMP2-producing cells. Either 1 × 10^5, 5 × 10^5, 1 × 10^6, or 5 × 10^6 BMP2-producing cells were injected into the C57BL/6 mouse quadriceps (n = 4 per dose). One day later the mice were euthanized and the limbs injected were frozen and serial sections prepared. These were stained with antibody to mouse PSs 1, 5, and 9 as well as an antibody to mouse NF. This was followed by reaction with secondary antibodies such that the PS was green and the NF red. The number of green cells per 10X field were counted. The average PS cell counts are presented versus dose of cells. One standard deviation unit above and below the average is shown. (p values: 1 × 10^5 to 5 × 10^5 cells, 0.04; 5 × 10^5 to 1 × 10^6 cells, 0.002; 1 × 10^6 to 5 × 10^6 cells, 0.007. (B): Single representative images of 10X fields of the experiment presented in panel (A) from three separate mice. (C): PS and TR are coexpressed in endoneurial cells. Wnt1CreErt: Ai9Tm mice were treated with tamoxifen on days −2, −1, and 0 and each injected with 5 × 10^5 BMP2-producing cells. Frozen sections were prepared and stained with anti-PS antibody followed by a detector antibody conjugated to Alexa fluor 488 (green), DAPI stain (blue). (D): PS is not expressed in the nerves of C57BL/6 mice that were not injected: (Da), NF; (Db), PS. C57BL/6 mice or that were injected with 5 × 10^5 cells transduced with AdEmpty: (Dc), NF; (Dd), PS. Quantification of the fluorescent photomicrographs in Figure 1 show that 95% – 9% of the PS⁺ cells express TR. Abbreviations: BMP2, bone morphogenetic protein type 2; NF, neurofilament; PS, phosphoSMAD; TR, Tomato red.

RESULTS

The Target of BMP2 is Limited to a Few Cells in the Endoneurium of Peripheral Nerves

To identify the cellular target of BMP2, mice were injected with varying numbers of AdBMP2-transduced cells (1 × 10^5, 5 × 10^5, 1 × 10^6, or 5 × 10^6), which resulted in varying the dose of BMP2 delivered. The entire hind limb was analyzed for cells responding to the BMP2 at 1 day after the initial injection. The results of immunostaining using an antibody that detects the complex of phosphorylated SMADs 1, 5, and 8 and therefore detects only signaling through receptors for bone morphogenetic proteins [19] showed a dose-dependent response (Fig. 1A). The number of positive cells per 10X field was determined by counting five fields on two slides prepared from the limb of each of the four mice that received a given dose. The results show that the average number of positive cells per group increased with the number of cells delivered up to a dose of 1 × 10^6 where it then plateaued (Fig. 1A). The dose-response curve of responder cells generated matches closely the increase

Human Tissues

Human tissues (three) were obtained from early heterotopic ossification, prior to radiographic evidence of mineralized bone assessed using muscle biopsy [6], from patients undergoing surgeries at Walter Reed National Military Medical Center (WRNMMC), through an approved IRB protocol (W374863). All human tissue transfers to Baylor College of Medicine (Olmsted-Davis) from WRNMMC (Forsberg) followed the approved Cooperative Research and Development Agreement (NCRADA-NMRC-13-9127) between BCM and the Department of the Navy. Tissues were shipped in formalin and processed as previously described [13]. The tissue was cut into pieces 2–15 mm in size. It was then fixed in buffered formalin, decalcified, processed, and embedded in paraffin. The tissues were sectioned (4 μm) and every fifth slide subjected to hematoxylin and eosin staining as previously described [18].
in the volume of the heterotopic bone versus BMP2 dose measured previously [20]. Mice that remained uninjected or were injected with cells transduced with AdEmpty were not found to contain PS$^+$ in the endoneurium (Fig. 1D).

Figure 2. TR signal appears in peripheral nerves 1 day after induction with BMP2 and in osteoblasts 7 days after induction. Wnt1CreER::Ai9Tm mice were injected with tamoxifen on days −2, −1, and 0 and on day 0 were also injected with BMP2-producing cells. On the first day after BMP2 induction the mice were euthanized, the hind limbs were harvested in sucrose, quick frozen, and 5 μm sections prepared. Sections were stained with an antibody to mouse NF and a detector antibody conjugated to Alexa fluor 488. (A) H&E, (B) SP7 (green), (C) TR and DAPI (blue), (D) TR, SP7 (green) and DAPI (blue). On the seventh day after BMP2 induction the mice were euthanized and sections prepared as described above. The slides were costained with an antibody against mouse SP7 (green). (E) H&E, F. SP7 (green), (G) TR and DAPI (blue) (H) shows TR merged with the image taken using polarized light (white). DAPI (blue). Control: Wnt1CreER::Ai9Tm were injected with BMP2-producing cells on day 0, but were not treated with tamoxifen. (I) H&E, (J) SP7 (green), (K) TR, (L) merger of TR and SP7 (green) + DAPI (blue). The inset in panel L shows an H&E of the bone nodule. Quantification of the fluorescent micrograph shown in Figure 2D shows that 100% of the SP7$^+$ cells in the endoneurium are positive for TR. Quantification of the fluorescent micrograph shown in Figures 2E and 2F show that 74% of the SP7$^+$ are positive for TR. Panels (M) through (T) were performed in C57BL/6 mice that were injected with either AdEmpty-transduced cells, which were euthanized on day 1, frozen sections prepared, and stained with (M) SP7 (green), (N) NF (red), (O) merger of DAPI (blue) and NF (red) or AdBMP2-transduced cells, which were euthanized on day 7, frozen sections prepared, and stained with (P) SP7 (green), (Q) Dlx5 (red), (R) Merger of SP7 (red) and Dlx5 (green), (S) BGLAP (green), (T) Merger of BGLAP (green) and SP7 (red). Abbreviations: BMP2, bone morphogenetic protein type 2; NF, neurofilament.

Representative images taken from tissues isolated 1 day after induction of HO from three separate mice are shown in Figure 1B. PS$^+$ cells (green) are costained with an antibody against the NF heavy chain (NF, red) that shows the location of...
peripheral nerves. In all cases, the PS\textsuperscript{+} cells are found in the neural endoneurium.

To determine if the PS\textsuperscript{+} cells in the endoneurium also express TR, HO was induced in Wnt1\textsuperscript{Cre\textsuperscript{ERT2};Ai9Tm} mice after induction with tamoxifen and tissues isolated 1 day later. The results (Fig. 1C) show that nuclear PS (green) is surrounded by the cytoplasmic TR signal in these cells. Quantification of the fluorescent photomicrographs in Figure 1 show that 96%\textpm{}9% of the PS\textsuperscript{+} cells express TR, and 4.3%\textpm{}1.2% of TR\textsuperscript{+} cells express PS on the first day after BMP2 induction.

**Figure 3.** SP7, Dlx5, and PS Expression is Present in Wnt1 (TR\textsuperscript{+}) Cells (A): Fluorescence-activated cell sorting (FACS) analysis of the TR\textsuperscript{+} cells. Wnt1\textsuperscript{Cre\textsuperscript{ERT2};Ai9Tm} mice were induced simultaneously (as described in the legend to Fig. 2) with only BMP2 (n = 6), with only tamoxifen (n = 6), or with BMP2 and tamoxifen (n = 6). After 4 days cells were isolated from the muscle surrounding the site of injection from individual mice in each group and subjected to FACS analysis. Bars are the average of each analysis and the lines through the bars are one standard deviation. \( p \) values were determined using a Student's \( t \) test. \( p < .005 \) (* and **). (B) FACS isolation of TR\textsuperscript{+} cells. Wnt1\textsuperscript{Cre\textsuperscript{ERT2};Ai9Tm} mice (n = 3) were induced with tamoxifen and BMP2 or with only BMP2 (n = 2). After 7 days' cells were isolated and subjected to FACS. Panel (Ba) shows the FACS profile of one of the mice induced with BMP2 only, which was used to set the gate for the FACS isolation of TR\textsuperscript{+} cells. Panel (Bb) shows the FACS profile of one of the mice induced with BMP2 and tamoxifen. The TR\textsuperscript{+} cells from the three mice induced with BMP2 and tamoxifen were pooled and used for the experiment described in 3(C) below. Likewise, the TR\textsuperscript{+} cells from these mice were also pooled. (C): Osteoblasts formed during HO are derived from the nerve. Wnt1\textsuperscript{Cre\textsuperscript{ERT2};Ai9Tm} (n = 3) were induced with BMP2 and tamoxifen as described above in Figure 3B above. The TR\textsuperscript{+} and TR\textsuperscript{+} cells from the three mice were each pooled separately. Then the TR\textsuperscript{+} and TR\textsuperscript{+} populations were deposited onto glass microscope slides by cytocentrifugation followed by reaction with an antibody against either SP7, Dlx5, or PS and a detection antibody conjugated to Alexa fluor 488 (green). Arrows indicate cells where there is overlap between the TR (red) and either SP7, Dlx5, or PS (green) stains. DAPI, blue. All images, magnification, \( \times 10 \).

Abbreviations: BMP2, bone morphogenetic protein type 2; TR, Tomato red.

**SP7\textsuperscript{+} Cells Expressing TR Are Initially Found in the Endoneurium of Peripheral Nerves and Are Then Found Associated With Bone at Later Times After Induction of HO**

To test whether SP7\textsuperscript{+} cells are contributing to osteoblast populations in HO, tissues were isolated from Wnt1\textsuperscript{Cre\textsuperscript{ERT2};Ai9Tm} mice treated with either tamoxifen or vehicle and then injected with BMP2-producing cells. SP7\textsuperscript{+} cells were observed coexpressing the TR reporter in the tissues isolated one day after BMP2 induction in mice receiving tamoxifen (Fig. 2A–2D). Similarly, tissues isolated 7 days after BMP2 induction also showed SP7\textsuperscript{+} staining in cells expressing TR (Fig. 2F, 2G). However, in the absence of tamoxifen there was no red signal observed in the sections (Fig. 2I–2K).

Finally, when C57BL/6 mice were injected with cells transduced with AdEmpty, euthanized one day later and frozen sections prepared and stained for SP7, there was no stain observed in peripheral nerves (Fig. 2, panels M–O). In addition, induction with BMP2-producing cells consistently produced bone formation within 7
days with osteoblasts expressing SP7, Dlx5, and BGLAP as shown in Figure 2P–2T.

**SP7, Dlx5, and PS Expression is Present in Wnt1 Cells and Is Absent in the Negative Population**

To confirm the changes in the reporter expression after induction with BMP2, three groups of Wnt1\(^{CRE}\):Ai9Tm mice were treated with tamoxifen (2 groups) or vehicle (1 group), and then on day 0 one of the tamoxifen-treated groups was injected BMP2-producing cells and the other group with PBS. The group treated with vehicle rather than tamoxifen was also injected with BMP2-producing cells. Four day after induction with BMP2 the tissue surrounding the site of early bone formation was digested and the isolated cells analyzed by FACS. This analysis (Fig. 3A) showed a population of 6.8% ± 1.0% TR\(^{+}\) cells 4 days after BMP2 induction relative to 2.2% ± 1.6% TR\(^{-}\) cells in the absence of tamoxifen but with injection of BMP2-producing cells and 1.2% ± 0.8% in the presence of tamoxifen but in the absence of BMP2-producing cells (\(p < .005\) for either comparison). To determine if SP7\(^{+}\) cells also expressed the red reporter, TR\(^{-}\) and TR\(^{+}\) populations were collected after FACS (Fig. 3B) and single cell suspensions of cells were deposited onto glass microscope slides by cytocentrifugation for subsequent immunofluorescence staining with antibody against mouse SP7. The result, shown in Figure 3C indicates that approximately 80% of the TR\(^{-}\) cells are also positive for SP7 further confirming that Wnt1-expressing cells from the endoneurium express the osteoblast specific transcription factor SP7. Analysis of cytocentrifuge preparations of the TR\(^{-}\) population revealed no SP7\(^{+}\) cells (Fig. 3B). Similarly, the results shown in Figure 3C also indicate that approximately 70% of the TR\(^{-}\) cells express the osteoblast transcription factor Dlx5 [22], while only approximately 15% of the TR\(^{-}\) cells are positive for PS on the fourth day after BMP2 induction. Neither SP7, Dlx5 nor PS are expressed in the TR\(^{-}\) population 4 days after BMP2 induction (Fig. 3C).

**TR Is Found in Chondroprogenitors and the Progenitors for Transient Brown Adipocytes**

Analysis of tissue at later times after BMP2 induction revealed areas of cartilage and fat with cells that were positive for TR. Tissues isolated from tamoxifen-treated Wnt1\(^{CRE}\):Ai9Tm mice 5 days after induction of bone formation were therefore immunostained for uncoupling protein 1 (UCP1) to determine if the TR\(^{-}\) cells observed in the tissues were associated with these brown adipocytes. The result (Fig. 4A) shows that a significant percentage of UCP1\(^{-}\) cells are also TR\(^{-}\).

**Figure 4.** The labeling pattern of pre-tBAT and pre-chondrocytes in HO indicate their progenitors arise from nerves. Wnt1\(^{CRE}\):Ai9Tm mice (\(n = 6\)) were treated with tamoxifen on day’s −2, −1, and 0 and then injected with 5 x 10⁶ BMP2 producing cells on day 0. On day 4, the mice were euthanized, the limbs around the injection site harvested and serial frozen sections prepared in sucrose to maintain the fluorescence of TR. These were then stained for either UCP1 (A) or Sox9 (C) and were then stained with a detector antibody conjugated to Alexa fluor 488 (green). Control: Wnt1\(^{CRE}\):Ai9Tm mice were injected with BMP2-producing cells as described above but without treatment with tamoxifen. Frozen sections were probed with antibodies against either UCP1 (B) or Sox 9 (D) followed by a detector antibody conjugated to Alexa fluor 488 (green), DAPI (blue). All images, magnification, ×20. Abbreviation: TR, Tomato red.
contribute to chondrogenic progenitors during HO. In all cases tissues isolated from the Wnt1CreErt:Ai9Tm mice treated with vehicle and BMP2 did not result in any detectable tomato red expression, although UCP1 and Sox 9 expression were observed (Fig. 4B, 4D, respectively).

Neural Stem Cells Labeled During Embryonic Differentiation Also Acquire the TR Label in Osteoblasts

The possibility exists that a nonneural cell expressing Wnt1 may be induced by BMP2. To rule this out experiments were performed in the Wnt1Cre2:Ai9Tm that was designed to track neural crest stem cells synthesized during embryonic development [17]. After 5 and 7 days of BMP2 induction TR+ cells were observed both in nerves and in osteoblasts on bone (Fig. 5). Peripheral nerves in Wnt1Cre2:Ai9Tm mice not injected with BMP2 were negative for TR (data not shown).

Analysis of Trauma-Induced HO in Humans Indicates Changes in Peripheral Nerves Similar to Those Identified in the Mouse Model

Human tissues encompassing early HO development were obtained from surgical discarded tissue through an Institutional Review Board-approved protocol. Histological analysis of the tissues was performed and results suggest significant similarities to the mouse. To locate peripheral nerves adjacent to the site of bone formation, tissues were subjected to hematoxylin and eosin staining. A representative photomicrograph was assembled using approximately 20 microscopic fields at ×4 magnification of tissues stained with hematoxylin and eosin (Fig. 6A). Results show many nerves immediately adjacent, and in some cases totally encased by, the newly formed bone (Fig. 6A).

Immunohistochemical staining for SP7 shows a significant number of cells positive for this master regulator (transcription factor) of osteoblastic differentiation, within the endoneurium of the nerves shown in Figure 6A. In this case the Schwann cell marker p75 was used to mark the nerve (Fig. 6B). Nerves obtained from normal tissue from patients not diagnosed with HO were negative for SP7 (Fig. 6C). However, in the tissue from patients with HO SP7+ cells were found on the surface of the bone directly adjacent to one of the nerves highly positive for SP7 (Fig. 6D).

DISCUSSION

In the mouse model of HO, BMP2-producing cells are injected into the mouse quadriceps inducing an almost synchronous series of events including remodeling of nerves (day 1), formation of transient brown fat (days 2–3), vessel formation (day 3), chondrogenesis (day 5, 6), and finally immature bone formation (day 6, 7) [11,24,25]. Although BMP2 is secreted from the cells injected into the soft tissues, surprisingly, the only cells that immediately respond are found within the endoneurium of peripheral nerves. Since these cells are behind the blood-nerve barrier [26], it is difficult to envision how BMP2 enters this location. However, the timing appears to be similar to induction of the neuro-inflammatory pathways previously described [12,14]. The lack of PS+ cells in the soft tissues outside the nerve may be a direct result of rapid binding of BMP2 by inhibitory binding proteins in the region [27]. Potentially the expression observed in the nerve may be a result of the restricted nature of the endoneurium, in that it lacks BMP2 regulatory binding proteins that could prevent receptor binding. The results suggest a novel mechanism by which BMP2 selectively signals to form bone. Several mechanisms may contribute to the neuroinflammation that serves to allow BMP2 to cross the blood-nerve barrier and bind to cells in the neural endoneurium. We [12] and others [14] have described several cells and mediators that may be involved in the neuroinflammatory pathway, including platelets and mast cells, as well as the mediators of pain, substance P and calcitonin gene-related peptide as well as the transient receptor potential cation channel, subfamily V, member 1 (TRPV1). MMP9 may also be intimately involved in opening of the barrier and is clearly elevated during the requisite time-frame [28].
Within the mouse endoneurium, only a handful of cells seem to express PS. These cells express TR, as determined by immunohistochemical analysis of the TR cells. SMAD signaling has recently been reported to be pulsatile because of the nuclear localization of SMAD4 that is required for the activity of PSs 1, 5, and 8. Additionally, PSs are rapidly degraded by both phosphatases and the proteasome [29]. During skull formation in the embryo, it is thought that BMP2 can directly upregulate Dlx5, leading to

Figure 6. Nerves from patients with heterotopic ossification (HO) contain cells that are positive for SP7 and a large number of cells are positive for PSs 1, 5, and 8. Cells on bone are similarly positive. Cells in nerves from patients not having HO are negative for SP7 and PSs. (A): Montage of H&E stained images, reconstructed digitally after taking the images at ×4 magnification and assembling them. (B): Staining of nerves sections from patients with HO were stained with either antibody against p75 (low affinity nerve growth factor receptor, red), SP7 (green), NF (red), and PS (green). (C): Control nerves. Nerves were identified in paraffin sections prepared from the tissue of patients (not diagnosed with HO). In the case of the first tissue sample the nerve was stained for DAPI (blue) and SP7 (green). For the second tissue sample the nerves were stained for NF (red) and PS (green). (D): Either SP7 (green) or PS (green) was visualized on the surface of the bone directly adjacent to nerves. Polarized light was used to assist in visualizing the bone (white). Abbreviations: NF, neurofilament; PS, phosphoSMAD.
expression of SP7 [30]. Previous studies demonstrated the expression of Dlx5 in the SP7⁺ endoneurial cells [7]. If BMP2 signaling is capable of launching this pathway, then it would suggest that progenitor cells in the endoneurium respond to BMP2 leading to trans- or osteogenic differentiation to osteoblasts. However, it is also possible that there is truly a limited, specialized population of cells responding to BMP2 in the nerve, and these cells either secrete factors or in some other manner induce the activation and differentiation of cells in the endoneurium.

The nature of the TR⁺ cells within the endoneurium is unclear. It has been reported that Schwann cells may differentiate to form progenitor cells in nerves [31]. Previous characterization of these cells demonstrated the presence of p75, a Schwann/neural stem cell marker, but also the lack of expression of any proteins associated with myelinating Schwann cells [7]. Another possibility is nonmyelinating Schwann cells. Although nonmyelinating Schwann cells enable the axons they surround to sprout more easily, their exact function is unknown [32]. In the embryo, Wnt1 is responsible for activation of neural stem cells [33] and if unopposed the activated stem cells become sensory nerves [34], yet if opposed by BMP2 these same cells differentiate to bone and other lineages [35].

To determine if osteoblasts in HO are derived from the SP7⁺ cells within peripheral nerves [7], a tamoxifen-regulated Wnt1-Cre recombinase lineage-tracing mouse (Wnt1CreERT:Ail9Tm) was used. The tamoxifen-regulated promoter, allowed the discernment of cells that become Wnt1⁺ in the adult from those that would potentially activate the reporter during embryogenesis. Thus, limited addition of the tamoxifen, particularly just preceding the addition of BMP2, allows tracing of cells newly positive for Wnt1 and therefore of neural origin [33]. Little or no red staining was observed in the nerve or other tissues in mice not given tamoxifen. Analysis of the tissues from mice receiving both tamoxifen and BMP2 showed the presence of the TR reporter only in the neural endoneurium one day after injection of BMP2-producing cells. At later days, the red reporter was observed in several cell types at the location of the newly forming bone. TR⁺ osteoblasts were associated with the newly forming bone, suggesting the neural origin for these cells. Additionally, the cells were observed in Sox9⁺ prechondrocytes and UCP1⁺ brown adipocytes.

This finding suggests the potential stem cell nature of these endoneurial cells. In the embryo, neural crest progenitors undergo an epithelial to mesenchymal transition, and these mesenchymal cells differentiate into multiple lineages [33]. In further support, characterization of TR⁺ and TR⁻ cells isolated by FACS showed that while the SP7⁺ cells were identified in the TR⁺ population, this population also included cells that were negative for SP7, suggesting there are other cell types derived from the endoneurial cells.

Our results also show that both tamoxifen treatment and BMP2 induction are required to generate TR⁺ cells in the Wnt1CreERT: Ail9Tm mouse, indicating that BMP2 is inducing Wnt1. Indeed, modulation of canonical Wnt signaling by BMP2 has been reported previously [36]. To confirm the coexpression of the red reporter and SP7, Wnt1TR positive and negative cells isolated by FACS were deposited onto glass microscope slides by cytocentrifugation, fixed and then subsequently immunostained for SP7. The data shows that the TR⁺ population contains no SP7⁺ cells indicating that all of the osteoblasts for HO are derived from the nerve. However, one possibility to account for this fact is that the injection of BMP2-producing cells is far away from other sources of osteoprogenitors, such as those in skeletal bone. Recently, in studies in the rat we showed that BMP2-producing cells injected far away from skeletal bone did not produce HO, but such cells, when injected close to skeletal bone, produced HO drawing progenitor cells from the peristium [37].

Histological analysis of the tomato red expression in tissues isolated from these mice, showed the presence of red expression in cells other than the osteoblasts. The tissues were further immunostained to identify the phenotype of these cells. Some of the transient brown adipocytes (UCP1⁺) as well as the early chondrocytes (Sox9⁺) also expressed the tomato red reporter, suggesting that they too are derived from peripheral nerves.

Comparison of the mouse findings to human HO induced by traumatic injury, revealed a striking similarity. The expression of PSs 1, 5, and 8, which are specifically induced by BMPs and no other TGFβ family members [29], was found in a large number of cells within the endoneurium. Not only was the expression in many more cells than in the mouse model, normal nerves from patients not suffering from HO were totally negative. Further, some PS⁺ cells were also SP7⁺ and were associated with the bone, indicating the presence of BMPs in the human tissues and suggesting, due to the short half-life of BMP proteins, their ongoing expression. This finding was surprising because many have reported the lack of BMP involvement this process [38], and criticism has been leveled at the use of BMP models for the study of HO. Perhaps the reduced number of cells expressing PS in the mouse compared to human nerve arises because of the nature of the mouse model. Previous studies have shown that the BMP2-producing cells are rapidly cleared from the tissues approximately 5 days after their injection [11, 24, 25]. Thus, the less robust response in mice may reflect the low-level short-term expression of BMP2. The analysis of human HO indicates the presence of SP7⁺ in the endoneurium similar to the finding in the mouse tissues. This suggests that peripheral nerves may also play a key role in HO induced by traumatic injury in humans. Since in combat casualties that suffer HO there is often traumatic brain injury [2,3], which compromises the blood-brain and blood-nerve barriers [2,39], such disruption may be part of the reason BMP2 can easily access the neural endoneurium in these patients.

A great deal of controversy has surrounded the search for the progenitors for bone formation during heterotopic ossification. On the surface, as noted above, it appears that groups have claimed widely disparate progenitors ranging from those from the
nerve described in this article, to endothelial cells [4], or progenitors residing in muscle [5]. However, as shown in the model of HO presented in Figure 7, each of these precursors or cells very much like them, is present during HO.

Detailed knowledge about the mechanism of the initiation of HO as well as the source of progenitors can help specify molecular targets for design of agents to prevent or treat HO. Further, confirmation that the mouse model mimics the human disease will provide a relevant model for characterizing the process as well as testing agents that may suppress it.

ACKNOWLEDGMENTS

This work was supported by grants from the Department of Defense (DAMD W81XWH-12-1-0274, “Diagnosis and Treatment of Heterotopic Ossification”) and the National Institutes of Health (NIH), National Institute of Arthritis and Musculoskeletal and Skin Diseases (R21AR061638, “Heterotopic Bone from Stem Cells in Nerve”; R21AR063779, “Function of Brown Adipocytes in Bone Formation”; R1AR065656, “Neural Mechanisms in Heterotopic Ossification”). One of the authors (EAS) was supported by a postdoctoral fellowship (NIH NIGMS K12 GM084897). We thank Rita Nistal her excellent work in preparation of the sections for histology and immunohistochemistry and Joel Sederstrom and the members of the Flow Cytometry Core at Baylor College of Medicine for excellence in providing assistance with flow cytometry analysis and sorting.

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AUTHOR CONTRIBUTIONS

E.O.-D., E.A.S., D.H., E.L.D., Z.L., T.A.D., J.A.F., and A.R.D.: Performing experiments, data analysis and interpretation, drafting and final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

This work was partially supported by BUMED Advanced Medical Development work unit # 604771N.0933.001.A0812. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government. Some of the authors are military service members or employees of the U.S. Government. This work was prepared as part of their official duties. Title 17 U.S.C. § 105 provides that “Copyright protection under this title is not available for any work of the United States Government.” Title 17 U.S.C. § 101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person’s official duties. The study protocol was approved by the Naval Medical Research Center Institutional Review Board in compliance with all applicable Federal regulations governing the protection of human subjects. All authors state that they have no potential conflicts of interest.

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