Identification of Progenitor Cells That Contribute to Heterotopic Skeletogenesis

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Background: Individuals who have fibrodysplasia ossificans progressiva develop an ectopic skeleton because of genetic dysregulation of bone morphogenetic protein (BMP) signaling in the presence of inflammatory triggers. The identity of progenitor cells that contribute to various stages of BMP-induced heterotopic ossification relevant to fibrodysplasia ossificans progressiva and related disorders is unknown. An understanding of the cellular basis of heterotopic ossification will aid in the development of targeted, cell-specific therapies for the treatment and prevention of heterotopic ossification.

Methods: We used Cre/loxP lineage tracing methods in the mouse to identify cell lineages that contribute to all stages of heterotopic ossification. Specific cell populations were permanently labeled by crossing lineage-specific Cre mice with the Cre-dependent reporter mice R26R and R26R-EYFP. Two mouse models were used to induce heterotopic ossification: (1) intramuscular injection of BMP2/Matrigel and (2) cardiotoxin-induced skeletal muscle injury in transgenic mice that misexpress BMP4 at the neuromuscular junction. The contribution of labeled cells to fibroproliferative lesions, cartilage, and bone was evaluated histologically by light and fluorescence microscopy. The cell types evaluated as possible progenitors included skeletal muscle stem cells (MyoD-Cre), endothelium and endothelial precursors (Tie2-Cre), and vascular smooth muscle (Smooth Muscle Myosin Heavy Chain-Cre [SMMHC-Cre]).

Results: Vascular smooth muscle cells did not contribute to any stage of heterotopic ossification in either mouse model. Despite the osteogenic response of cultured skeletal myoblasts to BMPs, skeletal muscle precursors in vivo contributed minimally to heterotopic ossification (<5%), and this contribution was not increased by cardiotoxin injection, which induces muscle regeneration and mobilizes muscle stem cells. In contrast, cells that expressed the vascular endothelial marker Tie2/Tek at some time in their developmental history contributed robustly to the fibroproliferative, chondrogenic, and osteogenic stages of the evolving heterotopic endochondral anlagen. Importantly, endothelial markers were expressed by cells at all stages of heterotopic ossification. Finally, muscle injury and associated inflammation were sufficient to trigger fibrodysplasia ossificans progressiva-like heterotopic ossification in a setting of chronically stimulated BMP activity.

Conclusions: Tie2-expressing progenitor cells, which are endothelial precursors, respond to an inflammatory trigger, differentiate through an endochondral pathway, contribute to every stage of the heterotopic endochondral anlagen, and form heterotopic bone in response to overactive BMP signaling in animal models of fibrodysplasia ossificans progressiva. Thus, the ectopic skeleton is not only supplied by a rich vasculature, but appears to be constructed in part by cells of vascular origin. Further, these data strongly suggest that dysregulation of the BMP signaling pathway and an inflammatory microenvironment are both required for the formation of fibrodysplasia ossificans progressiva-like lesions.

Clinical Relevance: These cell lineage tracing studies provide new insight into the cellular pathophysiology of heterotopic ossification. Therapeutic regulation of specific cell lineages involved in BMP-induced heterotopic ossification holds promise for the treatment of fibrodysplasia ossificans progressiva and possibly other more common disorders of heterotopic ossification.

Disclosure: In support of their research for or preparation of this work, one or more of the authors received, in any one year, outside funding or grants in excess of $10,000 from the International Fibrodysplasia Ossificans Progressiva Association, the Center for Research in FOP and Related Disorders, the Ian Cali Endowment, the Weldon Family Endowment, the Rita Allen Foundation, the Isaac and Rose Nassau Professorship of Orthopaedic Molecular Medicine, the Orthopaedic Research and Education Foundation Zachary Friedenberg Clinician-Scientist Award, and the National Institutes of Health (R01-AR41916 and R01-AG20911). Neither they nor a member of their immediate families received payments or other benefits or a commitment or agreement to provide such benefits from a commercial entity. No commercial entity paid or directed, or agreed to pay or direct, any benefits to any research fund, foundation, division, center, clinical practice, or other charitable or nonprofit organization with which the authors, or a member of their immediate families, are affiliated or associated.

Heterotopic ossification, the formation of bone in atypical locations, can lead to substantial disability. Enormous progress has been made in deciphering the molecular mechanisms of bone morphogenetic protein (BMP) signaling and its inductive role in heterotopic ossification[1-4]. However, in the forty years since the original description of the morphogenetic properties of demineralized bone matrix by Marshall Urist[5], little progress has been made in elucidating the lineage of endogenous responding cells responsible for the BMP-associated metamorphosis of soft connective tissue and skeletal muscle into heterotopic bone.

The genetic cause of fibrodysplasia ossificans progressiva, the most disabling form of heterotopic ossification in humans, was recently discovered to be a recurrent heterozygous missense mutation in the BMP type-I receptor, activin receptor IA/activin-like kinase-2 (ACVR1/ALK2), in all individuals with sporadic or familial inheritance of the classic form of the condition[3]. While a mutation in ACVR1, which results in dysregulation of BMP signaling, is the proximate genetic cause of fibrodysplasia ossificans progressiva, clinical observations implicate soft-tissue injury and the associated inflammatory response as important triggers of episodic disease flare-ups in genetically susceptible individuals[6-9]. A recent study showed that inflammatory cells of the hematopoietic lineage trigger heterotopic ossification in fibrodysplasia ossificans progressiva and in BMP-induced heterotopic ossification, although hematopoietic cells do not appear to contribute to the fibroproliferative, chondrogenic, or osteogenic stages of the heterotopic skeletal anlagen[9]. Rather, studies have suggested that cells associated with the vasculature are a likely source of responding cells in fibrodysplasia ossificans progressiva and in BMP-induced lesions[10,11]. Skeletal myoblasts are also a potential source of responding cells, given their osteogenic response to BMP signaling[12] and the muscle-associated anatomical location of fibrodysplasia ossificans progressiva lesions. Despite these findings, the precise cellular identity of the responding cells that form the heterotopic anlagen remains unknown.

Two mouse models of heterotopic ossification recapitulate characteristic histopathological features of fibrodysplasia ossificans progressiva[10,13] and many common acquired forms of heterotopic ossification. In one model, the direct injection of BMP/Matrigel into leg musculature or implantation at subcutaneous sites results in a robust osteogenic response that has been characterized in detail[10]. In the second model, the BMP4 gene is ectopically expressed at the neuromuscular junction under the control of the neuron-specific enolase (Nse) promoter, leading to progressive heterotopic ossification through an endochondral process[13]. In the present study, we investigate the cellular origin of fibroproliferative, chondrogenic, and osteogenic cells that contribute to the heterotopic endochondral anlagen in these animal models of dysregulated BMP signaling.

Materials and Methods

Transgenic Mice

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Universities of Pennsylvania and Connecticut. Nse-BMP4 transgenic mice, a gift from Drs. Lixin Kan and Jack Kessler (Northwestern University)[13], and *MyoD*^{iCre} knockin mice have been described[14]. SMMHC-Cre mice[15] were provided by Dr. Gary Owens (University of Virginia) and Tie2-Cre mice[16] were obtained from the Jackson Laboratory (Bar Harbor, Maine).

Cell-Specific Labeling with Use of Cre/loxP Recombination

Experimental mice were generated by crossing Cre transgenic mice with either R26R[17] or R26R-EYFP[18] reporter mice, which provide permanent, Cre-dependent expression of lacZ or EYFP, respectively (Fig. 1). A subset of the Cre;R26R mice was crossed with Nse-BMP4 transgenic mice to generate triple transgenic mice. Genotyping of the mice carrying the transgenes R26R, R26R-EYFP, Nse-BMP4, *MyoD*^{iCre}, SMMHC-Cre, and Tie2-Cre was performed as previously described[14-19]. All transgenic mice were screened to exclude the possibility of germline ectopic recombination[19,20].

BMP-Induced Heterotopic Ossification

In the BMP injection model of induced heterotopic ossification[10], growth factor-reduced Matrigel (BD Biosciences, Bedford, Massachusetts) was impregnated with recombinant human BMP2 (rhBMP2; provided by Genetics Institute [currently Wyeth, Cambridge, Massachusetts]) at a concentration of 2.5 μg/50μl and injected intramuscularly into the mid-belly of the tibialis anterior muscle of eight to twelve-week-old adult mice. Impregnated Matrigel solidifies at 37°C to form a localized source of BMP. Heterotopic tissues were recovered four days to two weeks following implantation for histochemical and immunohistochemical analysis.

In the Nse-BMP4 model, heterotopic ossification was induced by causing muscle injury by injecting 100 μl of 10 μM of cardiotoxin (Calbiochem, San Diego, California) in saline solution or in Matrigel into the quadriceps muscle. Contralateral limb controls included injection of Matrigel or saline solution without cardiotoxin to exclude the effect of damage related to the injection or the presence of saline solution or Matrigel[21]. Histochemical and immunohistochemical examination was performed at days 1, 4, 7, and 14 following injection.

Radiographic Evaluation

Whole-body radiographic images were made with use of Senographe DS technology (General Electric Medical Systems, Chalfont St. Giles, United Kingdom).

Tissue Preparation and Histology

Mouse muscle tissue was dissected, frozen, sectioned, and stained according to standard procedures[13]. Frozen blocks were stored at −70°C until sectioned for staining. Specifically, mouse tissue was dissected in phosphate-buffered saline solution, fixed by immersion in freshly prepared 2% formaldehyde in phosphate-buffered saline solution at 4°C for four hours, and then washed in three changes of phosphate-buffered saline solution at room temperature for one hour. Tissue was incubated in 10% sucrose in phosphate-buffered saline solution

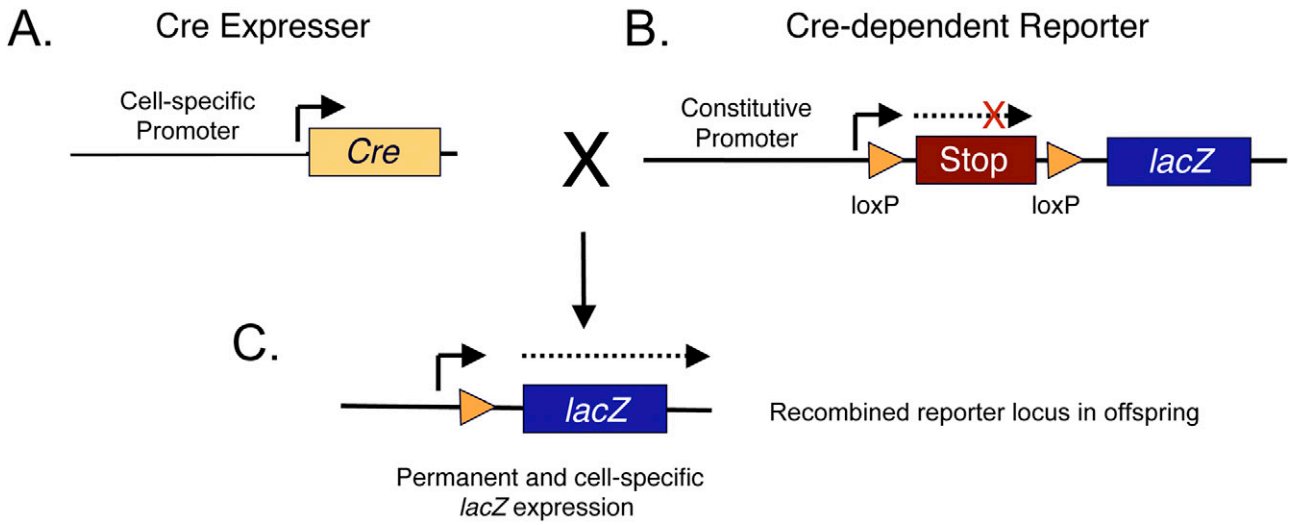

Fig. 1

Schematic of the Cre/loxP lineage tracing methodology. Transgenic mice expressing the Cre recombinase[48] under the control of a cell-specific promoter (A) are crossed to reporter mice (B) in which a reporter gene (e.g., *lacZ* in R26R mice) is separated from a constitutively active promoter by transcriptional stop sequences that are flanked by loxP sites, the target sequences recognized by Cre recombinase. Only cells of cell-specific Cre X loxP-lacZ offspring that express Cre undergo DNA excision of the stop sequences, resulting in transcription of the reporter gene (C). Note that loxP recombination at any point in a cell's developmental history results in permanent labeling of that cell and its daughters, regardless of whether Cre expression persists. In our experiments, the Cre-dependent reporters R26R[17] and R26R-EYFP[18] were used.

for thirty minutes at 4°C; then it was immersed in phosphate-buffered saline solution and 2 mM of $MgCl_2$, 30% sucrose, and 50% Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, California) at 4°C for two hours and it was frozen in O.C.T. Compound. Cryosections were cut at a thickness of 10 to 20 μm, and representative sections from throughout each lesion were mounted on Posi-Slides (Lab Storage Systems, St. Peters, Missouri).

Hematoxylin and eosin staining was performed with use of Harris Modified Hematoxylin and Eosin Y solution (Sigma-Aldrich, St. Louis, Missouri). Safranin-O staining was performed according to the instructions of the manufacturer (Sigma-Aldrich).

X-gal Staining

X-gal staining was performed as previously described[22], with minor modifications. Mounted tissue sections were fixed in 0.2% glutaraldehyde for ten minutes on ice, rinsed briefly in phosphate-buffered saline solution, and rinsed in detergent solution (0.05% NP-40 and 0.01% sodium deoxycholate in phosphate-buffered saline solution) for ten minutes at 4°C. Slides were washed in phosphate-buffered saline solution for ten minutes and were stained in X-gal staining solution overnight at room temperature in the dark. Some sections were counterstained with hematoxylin or eosin. Cells in representative sections were scored for β-galactosidase (β-gal) activity at a magnification of 100 to 400 times under bright-field illumination with either a Nikon Eclipse TE2000-U or Nikon E600 microscope (Nikon, Melville, New York).

Immunohistochemistry

For detection of endothelial markers, cryosections were cut at 8 μm and incubated in 2% horse serum diluted in phosphate-buffered saline solution for one hour. After several rinses in phosphate-buffered saline solution, the sections were incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-mouse CD144 (Santa Cruz Biotechnology, Santa Cruz, California), goat anti-mouse Tie2 (Santa Cruz Biotechnology), or rabbit anti-human von Willebrand Factor (Sigma-Aldrich). After three washes in phosphate-buffered saline solution, the sections were incubated with Alexa Fluor 488-conjugated donkey anti-rabbit or donkey anti-goat secondary antibodies (Invitrogen, Carlsbad, California) for one hour at room temperature. Controls included incubating sections in the absence of primary antibody or with nonspecific serum. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen), and sections were mounted with VECTASHIELD medium (Vector Laboratories, Burlingame, California). Slides were visualized and photographed with use of epifluorescence on a Nikon Eclipse TE2000-U microscope.

For enhancement of the EYFP (enhanced yellow fluorescent protein) signal, cryosections were rehydrated in two washes for five minutes each in phosphate-buffered saline solution and were incubated with blocking buffer (2% bovine serum albumin, 5% goat serum, and 0.1% Triton X-100 in phosphate-buffered saline solution) for forty-five minutes. Cryosections were incubated in a 1:500 dilution of rabbit anti-GFP (green fluorescent protein) antiserum (Invitrogen) in antibody dilution buffer (2% bovine serum albumin, 5% goat serum, and phosphate-buffered saline solution) for two hours, washed three times for five minutes each in phosphate-buffered saline solution, incubated in a 1:500 dilution of Alexa Fluor 488-conjugated goat anti-rabbit IgG (immunoglobulin-G) secondary antibody (Invitrogen) in antibody dilution buffer for forty-five

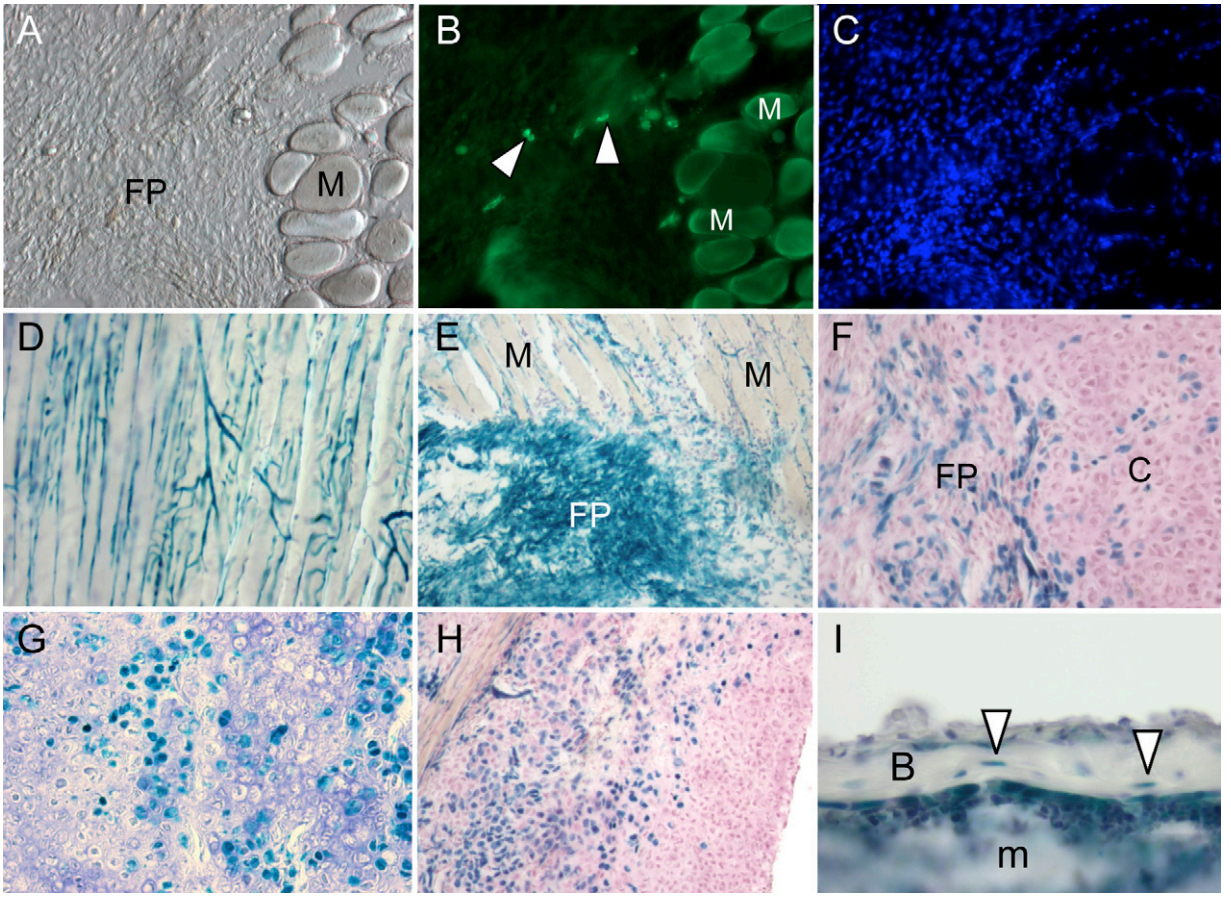

Fig. 2

Contribution of MyoD+ and Tie2+ cells to heterotopic ossification following intramuscular injection of BMP2. *A, B,* and *C:* *MyoD^{iCre}*;R26R-EYFP mice. *D* through *I:* Tie2-Cre;R26R mice. *A:* Phase image showing robust fibroproliferative response (FP) adjacent to skeletal muscle fibers (M), shown in transverse section, eight days after injection of BMP2. *B:* Fluorescence image of section in *A.* Only a few EYFP-labeled cells (examples at arrowheads) are present in the fibroproliferative lesion, whereas essentially all muscle fibers (M) are labeled. *C:* DAPI image showing the density of fibroproliferative cells. *D:* Longitudinal section of control, uninjected, muscle stained with X-gal. Tie2-Cre;R26R mice exhibit extensive β-gal labeling of the vasculature (blue staining). *E:* The majority of fibroproliferative cells (FP) four days after injection are intensely stained for β-gal. Longitudinal profiles of muscle fibers (M) are shown. *F:* At seven days after injection, the preosseous anlagen is composed of both fibroproliferative tissue (FP) and chondrogenic cells (C). In this section, representation of β-gal-labeled cells in the cartilage anlagen is lower than average. *G* and *H:* Typical examples of cartilage labeling from two additional mice, seven days after injection. Substantial regional variation in the extent of labeling is sometimes observed (compare left and right regions of H). *I:* New lamellar bone (B) fourteen days after BMP2 injection, showing β-gal-labeled osteocytes (two shown with arrowheads). Marrow elements (m) are intensely stained because of Tie2-Cre expression in hematopoietic lineages (original magnification, ×200).

minutes, washed three times for five minutes each in phosphate-buffered saline solution, and counterstained with 0.1 μg/mL DAPI in phosphate-buffered saline solution. Slides were cover-slipped with aqueous mounting media (Biomeda, Foster City, California) and were photographed with use of epifluorescence on a Nikon Eclipse TE2000-U microscope.

Source of Funding

The funding for this study was provided by the International Fibrodysplasia Ossificans Progressiva Association, the Center for Research in FOP and Related Disorders, the Ian Cali Endowment, the Weldon Family Endowment, the Rita Allen Foundation, the Isaac and Rose Nassau Professorship of Orthopaedic Molecular Medicine, the Orthopaedic Research and Education Foundation Zachary Friedenberg Clinician-Scientist Award, and the National Institutes of Health (R01-AR41916 and R01-AG20911).

Results

Skeletal and Smooth Muscle Progenitors Contribute Minimally to BMP2-Induced Heterotopic Ossification

Cre/loxP lineage tracing methodologies were used to investigate cellular sources of heterotopic ossification (Fig. 1).

TABLE I Cell Lineage Contributions to the Heterotopic Endochondral Anlagen*

Promoter	Cell Lineage	Heterotopic Skeletal Anlagen Stages†		
		Fibroproliferative	Chondrogenic	Osteogenic
MyoD	Skeletal muscle	<5	<1	ND
SMMHC	Vascular smooth muscle	ND	ND	ND
Tie2	Endothelial	40-50	40-50	40-50

*Combined data from both BMP2 injection and Nse-BMP4 models. The numbers represent the percent contributions of labeled cells to each stage of heterotopic ossification and are averages from at least three animals at each stage for each model. The numbers are approximations as there was considerable variability within and between histological sections (Figures 2 and 4 show typical examples). †ND indicates labeled cells were not detected.

Several candidates have been proposed as potential cells of origin of heterotopic ossification, including cells of the skeletal muscle lineage and the vasculature[11]. We used *MyoD*^{iCre} knockin mice, in which the *Cre* gene was knocked into the *MyoD* locus, to test the contribution of skeletal muscle cells to all stages of heterotopic ossification following intramuscular injection of BMP2. *MyoD* is a regulatory gene expressed exclusively in skeletal muscles, and all skeletal muscles are efficiently labeled in *MyoD*^{iCre};R26R mice[19]. Importantly, muscle stem cells (satellite cells) also are labeled in these mice because of the activity of the *MyoD* locus in satellite cell progenitors. Despite the expression of osteogenic markers in skeletal myoblasts exposed to exogenous BMPs[12], labeled cells were observed only occasionally in BMP2-induced lesions in *MyoD*^{iCre};R26R-EYFP mice (Fig. 2, *A, B,* and *C;* Table I). The maximal contribution of labeled cells to the early fibroproliferative lesion was approximately 5% in histological sections, with the average contribution being substantially lower (Fig. 2, *A, B,* and *C*). These labeled cells likely represent satellite cells that were activated by the intramuscular injection, which produces a minor injury. Labeled cells were found rarely in heterotopic cartilage, and no labeled osteogenic cells were observed. Mobilization of the satellite cell population by injection of cardiotoxin one day prior to BMP2 injection did not increase the contribution of myogenic cells to heterotopic cartilage or bone (data not shown). These data show that skeletal myogenic cells do not significantly contribute to BMP2-induced heterotopic ossification in vivo.

Fibroproliferative cells of early fibrodysplasia ossificans progressiva lesions express multiple smooth muscle lineage markers, suggesting a possible origin of lesional cells from vascular smooth muscle[11]. We tested whether vascular smooth muscle cells directly contribute to BMP2-induced heterotopic ossification in SMMHC-Cre;R26R mice. SMMHC (smooth muscle myosin heavy chain) is an early marker of vascular smooth muscle, and SMMHC-Cre expression closely matches endogenous SMMHC expression[15]. The vascular smooth muscle of adult mice is efficiently and permanently labeled in mice harboring the SMMHC-Cre transgene and a Cre-dependent reporter[15]. Labeled cells did not contribute to any stage of heterotopic ossification following BMP2 induction (Table I), excluding vascular smooth muscle as an important source of osteogenic progenitors in this model system.

Tie2+ Precursors Contribute to All Stages of BMP2-Induced Heterotopic Ossification

Next, we turned our attention to endothelium and endothelial precursors. Endothelium and endothelial precursor cells were labeled with use of Tie2-Cre transgenic mice[16], in which Cre expression is driven by regulatory elements of the *Tie2* gene (also known as Tek). Tie2, a receptor tyrosine kinase for angiopoietins, plays a critical role in the development of the embryonic vasculature and is ubiquitously expressed in early endothelial precursors during development and postnatal tissue repair[23-28]. In the adult, the skeletal muscle vasculature is extensively labeled in Tie2-Cre;R26R adult mice (Fig. 2, *D*), owing to labeling of essentially all CD31+ endothelial cells (unpublished observations). In contrast to the results with *MyoD*^{iCre} and SMMHC-Cre mice above, Tie2-expressing cells were a major contributor to fibroproliferative, chondrogenic, and osteogenic stages of heterotopic endochondral ossification in response to BMP2 implants (Fig. 2, *D* through *I*). Considerable variation was observed within and between histological sections in the degree of contribution of labeled cells to the fibroproliferative lesion and skeletal anlagen (Fig. 2, *F, G,* and *H*). Rarely, entire regions of a BMP2-induced lesion were devoid of labeled cells, whereas regions of labeling approaching 100%, even of the same growth, were also observed. This variation does not appear to reflect heterogeneity in the efficiency of labeling the endothelial population. A more likely possibility is that multiple cell types, perhaps regionally localized, contribute to heterotopic ossification. We note that while hematopoietic stem cells express Tie2[29,30], recent bone marrow transplantation studies have shown that cells of the hematopoietic system do not contribute to fibroproliferative, chondrogenic, or osteogenic stages of BMP-induced heterotopic ossification[9].

Cardiotoxin-Induced Injury of Skeletal Muscle Stimulates and Synchronizes Heterotopic Ossification in Nse-BMP4 Transgenic Mice

In order to model the type of injury-induced lesions experienced by individuals who have fibrodysplasia ossificans progressiva, we

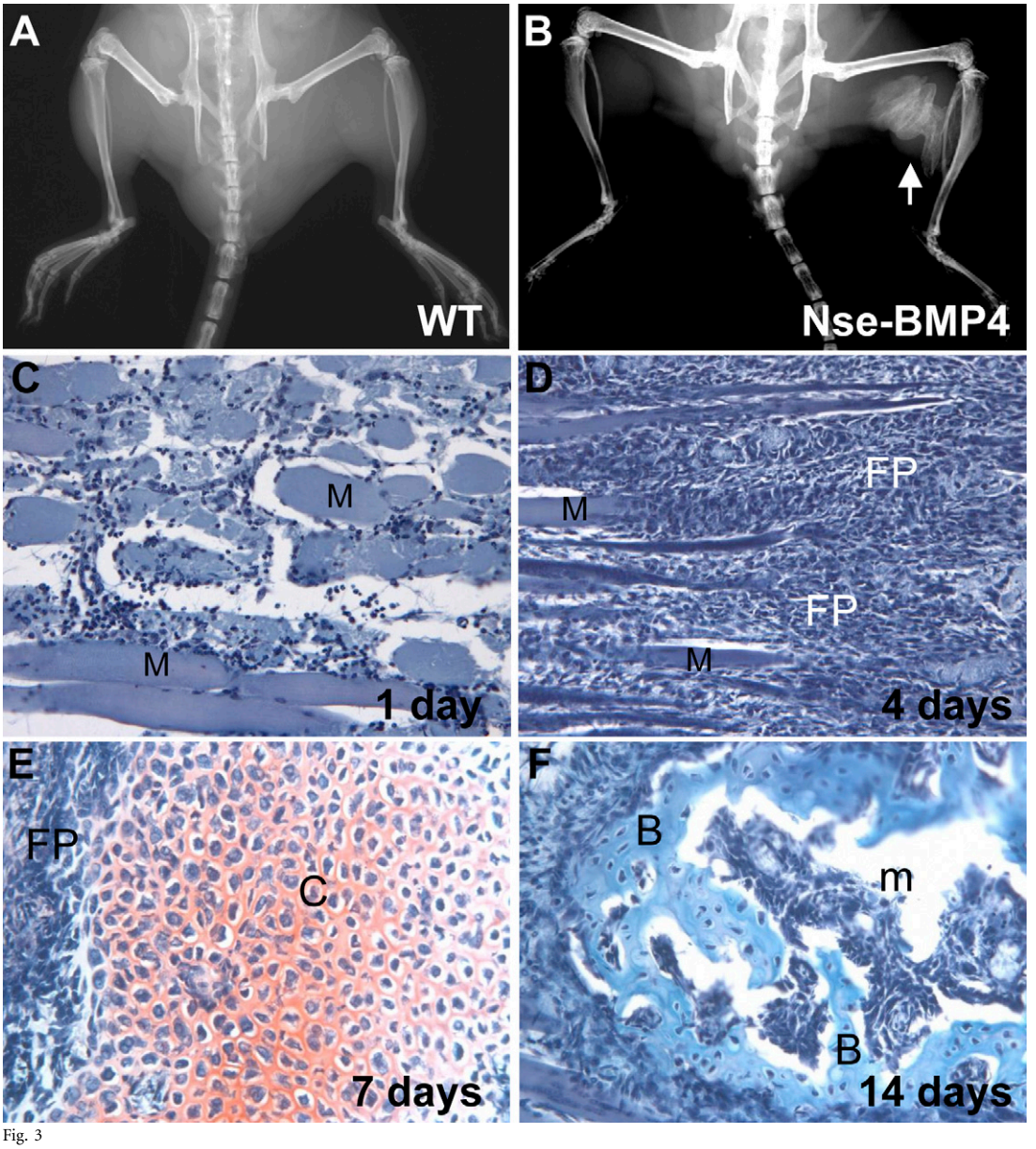

Fig. 3

Cardiotoxin injury of skeletal muscle stimulates and synchronizes heterotopic ossification in Nse-BMP4 transgenic mice. *A* and *B:* Radiographs of wild-type (A) and Nse-BMP4 (B) mice made three weeks after cardiotoxin injection. The white arrow indicates heterotopic ossification, which was observed only in the Nse-BMP4 transgenic mouse. Control injections with phosphate-buffered saline solution did not result in heterotopic ossification. *C* through *F:* Histological sections of lesions from cardiotoxin-injected Nse-BMP4 mice at one, four, seven, and fourteen days after injection, showing the morphology and features of heterotopic ossification. *C:* Muscle degeneration and lymphocytic infiltration (small round cells) is apparent at day 1. Two muscle fibers (M) are marked. *D:* A robust fibroproliferative response is apparent by four days. Fibroproliferative tissue (FP) is interspersed among the remaining muscle fibers (M). *E:* Section showing extensive cartilage differentiation (C) at seven days. Fibroproliferative cells (FP) are also visible in this field. *F:* At fourteen days, heterotopic bone (B; light blue) with marrow elements (m; dark blue) is present. (Safranin O staining; cartilage matrix is orange to red, and nuclei are blue; original magnification, ×200).

used a transgenic animal model of progressive heterotopic ossification in which BMP4 is misexpressed at the neuromuscular junction[13]. Previous studies have revealed that Nse-BMP4 transgenic mice do not develop heterotopic ossification spontaneously before two months of age and that there is wide variability among individual littermates in the onset of spontaneous

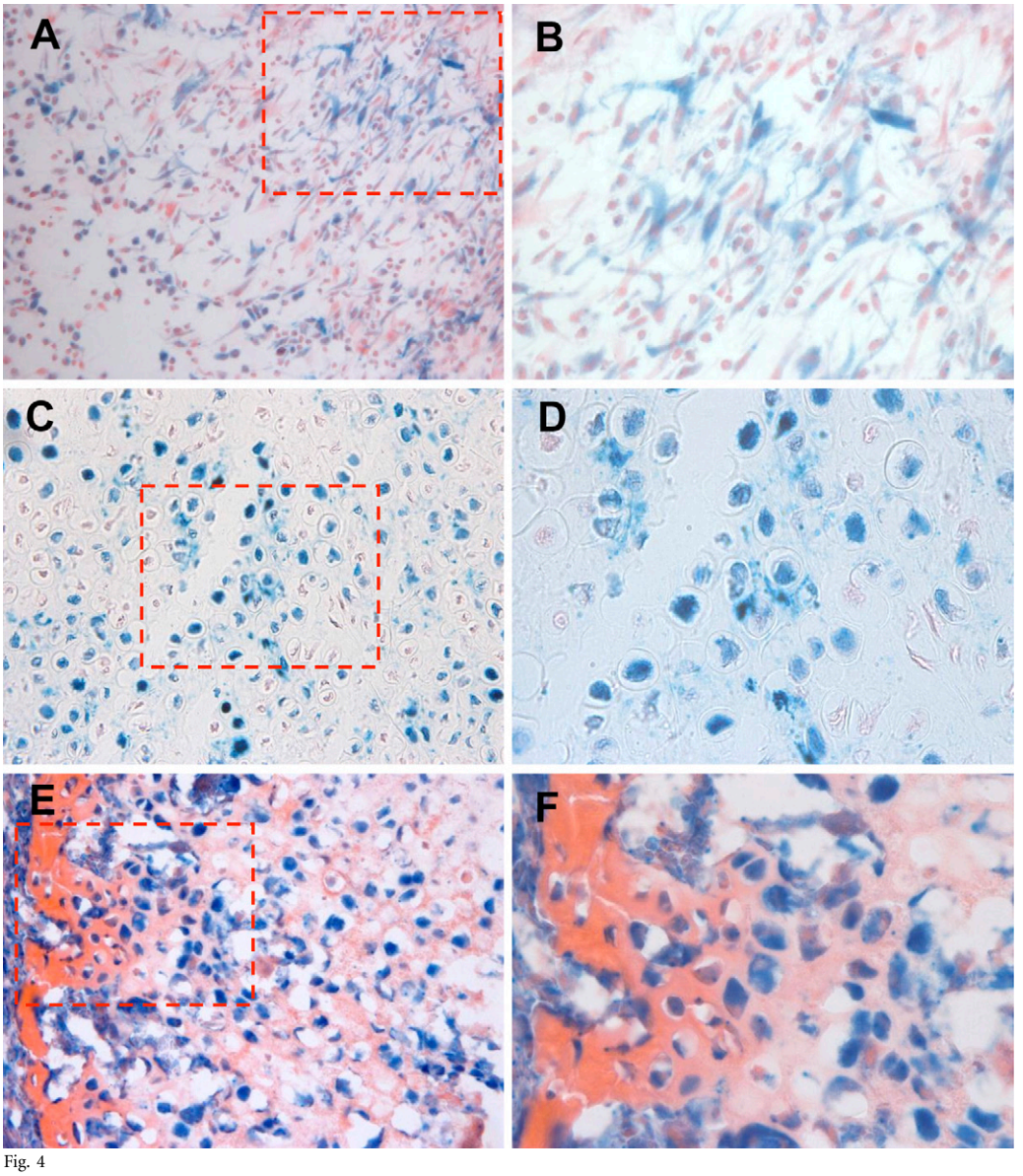

Fig. 4

Tie2+ cells contribute to all stages of heterotopic ossification after cardiotoxin-induced muscle injury in Tie2-Cre;R26R;Nse-BMP4 transgenic mice. *A* and *B:* Section of fibroproliferative tissue four days after cardiotoxin injection into the quadriceps muscle. X-gal staining revealed many β-gal-positive cells (blue). Labeled cells include both rounded lymphocyte-like cells and fibroblastic cells. Panel *B* represents a higher magnification of the boxed region in *A*. *C* and *D:* Low and high-power magnification images of chondrogenic area developing in muscle seven days after cardiotoxin injection. Approximately 50% of cartilage cells are X-gal stained (blue). *E* and *F:* Low and high-power magnification images of developing bone fourteen days after cardiotoxin injection. The majority of osteocytes were positive for β-gal. All sections were counterstained with eosin (original magnification, ×100 for A, C, and E and ×400 for B, D, and F).

heterotopic ossification, even though they have the same genetic background and live in a similar environment[13]. Skeletal muscle injury is a powerful trigger of heterotopic ossification in individuals who have fibrodysplasia ossificans progressiva. Similarly, we reasoned that an inflammatory skeletal muscle injury might trigger heterotopic ossification in Nse-BMP4 transgenic mice earlier and more reproducibly than would occur spontaneously.

We tested the muscle injury hypothesis by injecting cardiotoxin, a potent inflammatory stimulus and toxin that causes muscle degeneration, intramuscularly into the quadriceps muscle of one-month-old Nse-BMP4 transgenic mice. Three weeks after muscle injury, all ten cardiotoxin-injected limbs of Nse-BMP4 mice had heterotopic ossification develop, while no limb in any of the sham-injected controls had

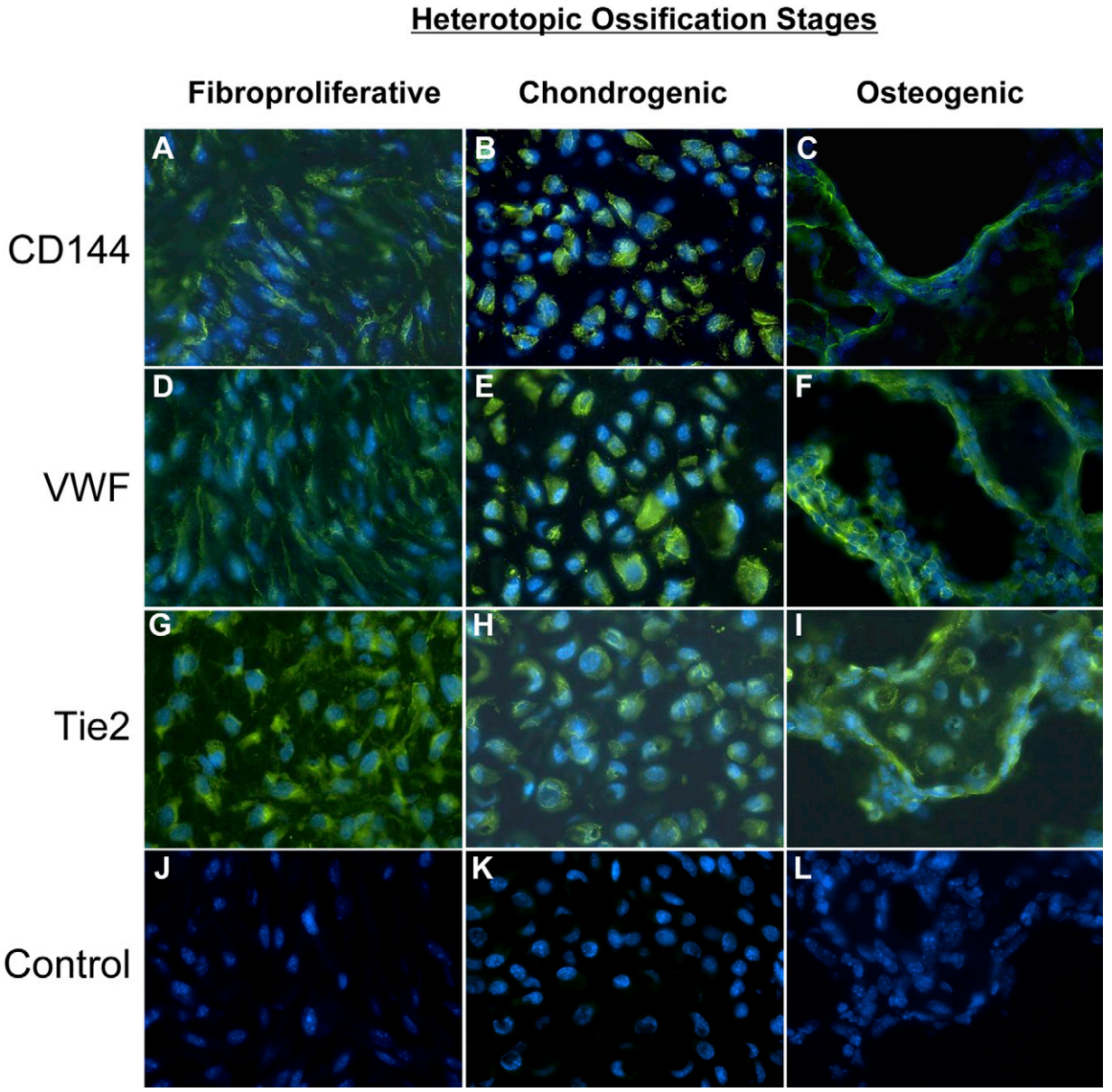

Fig. 5

Endothelial markers are expressed at all stages of the endochondral anlagen in BMP4-associated heterotopic ossification. Stages of endochondral ossification: fibroproliferative (A, D, G, and J), chondrogenic (B, E, H, and K), and osteogenic (C, F, I, and L). Sections were indirectly labeled for CD144 (A, B, and C), von Willebrand Factor (D, E, and F), and Tie2 (G, H, and I). Control sections in which nonspecific serum replaced the primary antibody (J, K, and L) showed no immunoreactivity. Nuclei (blue) were stained with DAPI, and immunostaining and DAPI images were merged. Each stage is a mixture of immunoreactive and nonreactive cells, consistent with the lineage tracing results (Figs. 2 and 4) (original magnification, ×600).

heterotopic ossification develop (Fig. 3, *A* and *B*). Histopathological evaluation at different time points revealed that the major sequential pathological changes in heterotopic ossification in cardiotoxin-injected Nse-BMP4 mice were essentially identical to those observed in fibrodysplasia ossificans progressiva lesions or in experimental lesions following local injection of BMP4 into mouse skeletal muscle[10,31]. In all cases, we saw an intense perivascular mononuclear cell infiltrate consisting of macrophages and lymphocytes, muscle degeneration, and an intense fibroproliferative response, followed by robust chondrogenesis and finally osteogenesis with heterotopic marrow elements (Fig. 3, *C* through *F*). Control mice showed nearly complete regeneration and reestablishment of normal muscle architecture during the time course of the experiment, and they exhibited no evidence of heterotopic ossification (data not shown). These studies indicate that cardiotoxin-induced

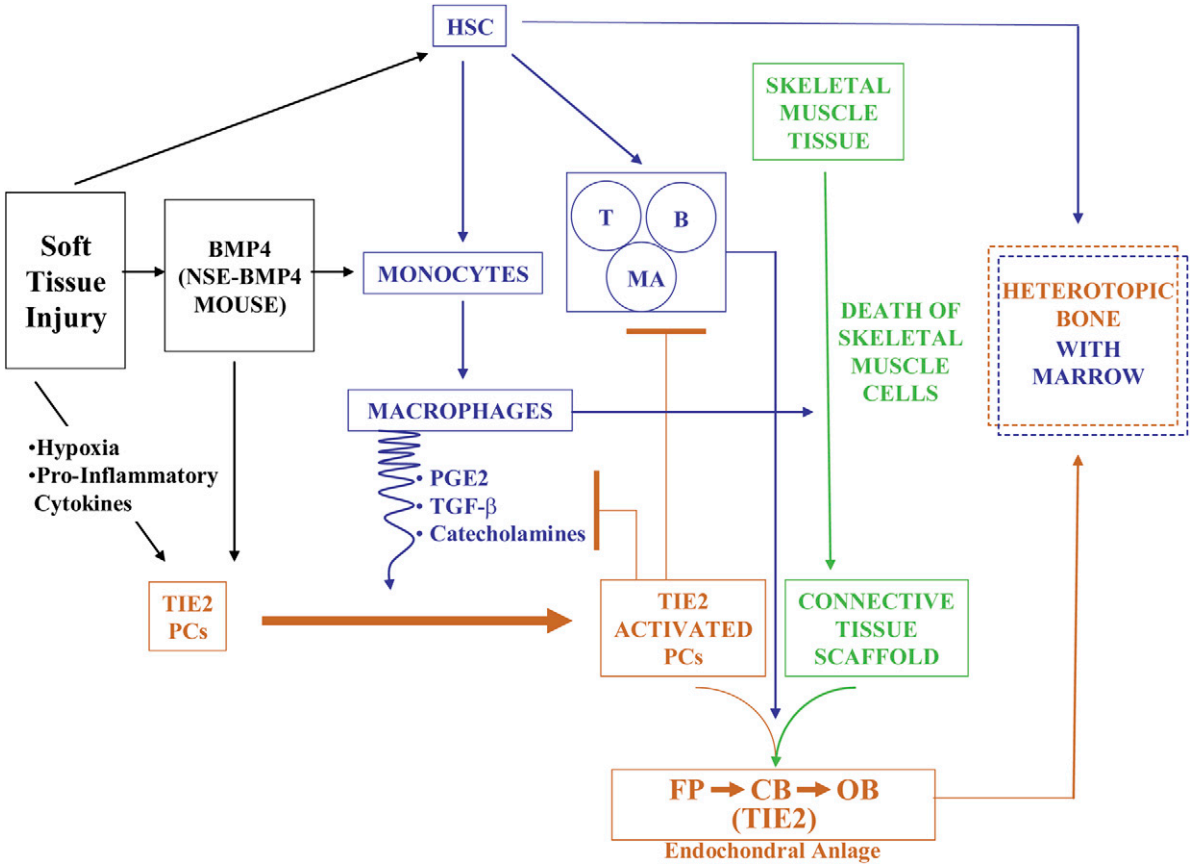

Fig. 6

Working model of BMP-associated heterotopic ossification. Injury to skeletal muscle and connective tissue leads to monocyte invasion, macrophage activation, tissue hypoxia, and upregulation of inflammatory cytokines and osteogenic factors including BMPs that recruit Tie2-expressing progenitor cells to form the heterotopic anlagen[35,42,49-51]. Wound hypoxia and inflammatory cytokines contribute to the angiogenic response in wound-healing, at least in part, by upregulating the expression of Tie2 mRNA and protein in these endothelial cells[42]. Hypoxia-related pH changes may further sensitize fibrodysplasia ossificans progressiva cells to ambient levels of BMPs[52], which further upregulates Tie2 expression and subsequent endothelial cell mobilization and migration[53]. The inflammatory reaction to muscle injury, the secretion of BMPs, and the cross-talk between cells of the innate and adaptive immune system stimulate the induction and propagation of an ectopic skeletal element. The blue lines indicate hematopoietic-derived pathways; brown lines, connective tissue progenitor-derived pathways; black lines, basal and very early post-traumatic conditions; green lines, muscle-derived pathways; blunt-end lines, inhibitory pathways; and arrows, stimulatory pathways. PC = progenitor cells, HSC = hematopoietic stem cells, T = T-cells, B = B-cells, MA = mast cells, FP = fibroproliferative cells, CB = chondroblasts, OB = osteoblasts, PGE_2 = prostaglandin E_2, and TGF-β = transforming growth factor-beta.

skeletal muscle injury in Nse-BMP4 mice stimulates an intense early inflammatory response and induces and synchronizes heterotopic ossification at an earlier time than would occur spontaneously. Importantly, muscle injury and associated inflammatory changes were sufficient to trigger fibrodysplasia ossificans progressiva-like heterotopic ossification in a setting of chronically stimulated BMP activity.

Tie2+ Precursors Contribute to All Stages of Injury-Induced Heterotopic Ossification in the Nse-BMP4 Transgenic Mouse Model

Next, we tested whether a Tie2 progenitor population contributes to heterotopic ossification following muscle injury in Nse-BMP4 mice, a model that, compared with the implant model, better represents the pathophysiology of fibrodysplasia ossificans progressiva. Cardiotoxin-induced muscle injury of Tie2-Cre;R26R;Nse-BMP4 mice resulted in robust labeling of all stages of heterotopic endochondral ossification (Fig. 4, Table I). On the average, approximately 50% of the fibroproliferative cells, chondrocytes, and osteoblasts were labeled in this model, similar to the degree of Tie2 precursor contribution observed in the BMP2-injection model described above (Fig. 2). Interestingly, immunohistochemical analyses revealed that the majority of cells of these heterotopic anlagen actively express Tie2 protein, as well as the endothelial markers, von Willebrand Factor and CD144 (Fig. 5). Expression of these markers was

also confirmed in the BMP injection model (data not shown). Taken together, these data suggest that endothelial cells from mature or immature vessels are a major source of progenitors to all stages of heterotopic ossification in response to BMP2/4 signaling, and that these cells continue to express vascular markers during the morphogenetic process.

Discussion

Despite intensive efforts, the lineages of progenitor cells that respond to BMP signaling and directly contribute to the formation of ectopic bone, as originally described by Urist[5], have remained elusive. We used Cre/loxP lineage tracing methods in the mouse to identify the origin of cells that are directly responsible for ectopic skeletogenesis. In the present study, we show that soft-tissue injury and associated inflammatory changes induce and synchronize endochondral heterotopic ossification in transgenic mice that misexpress BMP4 at the neuromuscular junction[13]. Intramuscular injection of BMP2 or BMP4 protein is also sufficient to elicit ectopic skeletogenesis, and both models of heterotopic ossification closely resemble the progression of histopathological events observed in patients with fibrodysplasia ossificans progressiva[10]. In both models, we demonstrated that progenitor cells that express Tie2 in their developmental history contribute substantially to every stage of the endochondral anlagen, including the fibroproliferative, chondrogenic, and osteogenic stages.

In addition to Tie2 expression in endothelial cells, in which it regulates growth, remodeling, and maintenance of the vasculature, Tie2 is also expressed by hematopoietic stem cells[29,30], raising the possibility that Tie2+ progenitors that participate in heterotopic ossification arise from the hematopoietic system and not from endothelium of the local vasculature. Recent studies on bone marrow transplantation, however, while pointing to the importance of hematopoietic cells in triggering heterotopic ossification, have clearly shown that the preosseous skeletal anlagen is derived from cells of nonhematopoietic origin[9]. These data strongly suggest that the labeled cells in heterotopic lesions of Tie2-Cre;R26R mice arise from the endothelium of the local vasculature, in response to injury and BMP signaling.

BMP receptors are highly expressed on endothelial cells in vivo, and the BMP-Smad pathway potently activates the endothelium[32]. In addition, BMPs have the ability to redirect the differentiation of connective tissue progenitor cells[33-35] to orchestrate an endothelial-to-mesenchymal transition in these cells, often through inflammatory cell intermediates[36-40]. Interestingly, misexpression of constitutively active ACVR1/ALK2, a BMP type-I receptor and the gene mutated in fibrodysplasia ossificans progressiva, is sufficient to stimulate an endothelial-to-mesenchymal transformation in endothelial cells of the heart[41]. Notably, BMP4, as well as hypoxia and inflammatory cytokines—conditions and factors that are present in the earliest preosseous lesions of heterotopic ossification—upregulate Tie2 in endothelial cells, which contributes to the angiogenic response[39,42]. The ongoing expression of Tie2 and other endothelial markers at all stages of BMP-induced ossification is likely a response to these local environmental cues and is entirely consistent with previous reports in two animal models of bone regeneration and fracture callus formation[43]. Thus, the heterotopic skeleton is not only supplied by a robust vasculature, it is actually derived from and formed in part by vascular cells. Of note, normal developing cartilage and bone of the embryonic skeleton are not derived from Tie2+ progenitors (unpublished observations), suggesting key differences in the etiology of normotopic and heterotopic bone.

Clinical studies have identified inflammatory cells in the earliest fibrodysplasia ossificans progressiva lesions[7,11], and long-term quiescence of fibrodysplasia ossificans progressiva following chronic immune suppression has been noted[8,9]. Cunningham et al.[44] showed that BMPs have profound effects on the recruitment of monocytes, known precursors of tissue macrophages. Subsequent studies have shown that BMP receptors are robustly expressed on monocytes and tissue macrophages[45]. Importantly, previous studies have identified macrophage recruitment to the early injury site following muscle trauma in the Nse-BMP4 mice[13]. These data strongly suggest that macrophages are involved in the induction of injury-induced heterotopic ossification in Nse-BMP4 transgenic mice. Our working model of BMP4-induced heterotopic ossification, which integrates the inflammatory reaction to muscle injury, the secretion of BMPs, and the cross-talk between cells of the innate and adaptive immune system, is shown in Figure 6. Importantly, our study shows that muscle injury and associated inflammatory changes are sufficient to trigger fibrodysplasia ossificans progressiva-like heterotopic ossification in a setting of chronically stimulated BMP activity.

In both models of heterotopic ossification used in the present study, approximately 50% of the heterotopic chondrogenic and osteogenic cells were derived from Tie2+ progenitor cells. Since nearly 100% of endothelium is labeled in Tie2-Cre mice (unpublished observations), incomplete labeling of the BMP-induced skeletal anlagen is not a consequence of inefficient labeling of the Tie2 lineage. Rather, these data indicate that at least one additional progenitor population (Tie2-negative) can respond to BMP2/4 and injury signals and can participate in ectopic skeletogenesis. Among possible progenitors, satellite cells—muscle stem cells responsible for muscle repair—were particularly attractive candidates. Satellite cells in culture downregulate the myogenic program and express osteogenic markers in response to BMP signaling[12,46] (unpublished observations). Further, fibrodysplasia ossificans progressiva lesions and BMP-induced heterotopic ossification are restricted to skeletal muscle and associated soft tissues, raising the possibility that ectopic skeletogenesis is mediated by stem and/or progenitor cells specific to muscle tissue. Surprisingly, however, satellite cells contributed minimally to BMP-induced heterotopic lesions, even when the satellite cell pool was activated by cardiotoxin-induced muscle injury prior to administration of BMP2. At the time of writing, we were evaluating other stem cell sources in muscle tissue[47] for their osteogenic capacity.

Previous studies have shown that early fibroproliferative lesional stromal cells of fibrodysplasia ossificans progressiva

and BMP-induced lesions express multiple smooth muscle lineage markers[11]. Smooth muscle marker expression could reflect a vascular smooth muscle origin or could result from de novo activation of smooth muscle markers in lesional cells originating from other sources. Using SMMHC-Cre transgenic mice[15], we did not observe a contribution of vascular smooth muscle cells to any stage of BMP-induced heterotopic ossification. The origin of smooth muscle-like cells in heterotopic lesions, therefore, remains to be determined. One candidate is the pericyte, a smooth muscle-like mural cell of the microvasculature that exhibits multilineage differentiation capacity[47]. Importantly, pericytes exhibit chondrogenic and osteogenic differentiation potential in some settings[47], making them an excellent candidate progenitor of heterotopic ossification. Pericytes are difficult to evaluate at present, however, because pericyte-specific reagents for lineage tracing have not yet been developed.

The discovery that Tie2-expressing vascular cells contribute to the heterotopic endochondral anlagen will aid in the development of cell-specific therapeutic strategies to treat fibrodysplasia ossificans progressiva and more common conditions of BMP-associated heterotopic endochondral ossification. Given the prevalence of BMP signaling in diverse cellular processes, the ability to target specific cell populations is of primary importance in order to minimize collateral effects. Mouse models of fibrodysplasia ossificans progressiva should prove invaluable for testing treatment modalities and drug discovery. In this regard, while the two mouse models of heterotopic ossification used herein recapitulate important clinical features of fibrodysplasia ossificans progressiva, they are not perfect models for the condition. We are currently developing gene-targeted mice that harbor the same activating mutation in the ACVR1/ALK2 BMP receptor that causes classic fibrodysplasia ossificans progressiva in all affected individuals[3]. Studies in those mice will enable a more comprehensive understanding of the cellular triggers and the repertoire of responsive progenitor cells in fibrodysplasia ossificans progressiva and will provide a systematic background for the development of the most appropriate medications and treatment for the condition. ■

NOTE: The authors thank Bob Caron for his invaluable technical assistance. The study was received in original form August 8, 2008 and accepted September 25, 2008.

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