Molecular Pathogenesis of Genetic and Inherited Diseases

Transgenic Mice Overexpressing BMP4 Develop a Fibrodysplasia Ossificans Progressiva (FOP)-Like Phenotype

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Fibrodysplasia ossificans progressiva (FOP) is a rare hereditary connective tissue disease characterized by progressive postnatal heterotopic bone formation. Although the genetic defects of FOP are not known, several lines of evidence have suggested that bone morphogenetic protein-4 (BMP4) may be involved in the pathophysiology. Nevertheless BMP4-transgenic mice have previously failed to develop the disorder and there has been no good animal model of the disease. Here, we report that a unique transgenic mouse line that overexpresses BMP4 under control of the neuron-specific enolase (NSE) promoter develops a FOP-like phenotype. Mating of these animals with transgenic animals that overexpress the BMP inhibitor noggin prevents the disorder, confirming the role of BMP4 in the pathogenesis of the disease. Heterotopic bone formation in these animals appears to follow the classic endochondral ossification pathway. Sex-mismatched cell transplantation experiments indicate that multiple cell sources contribute to the heterotopic ossification. This remarkable animal model provides a unique opportunity to further study the role of the BMP signaling pathway in heterotopic ossification and to improve our understanding of the clinical aspects of FOP. (*Am J Pathol* 2004, 165:1107-1115)

Fibrodysplasia ossificans progressiva (FOP), also known as myositis ossificans progressiva, is a autosomal dominantly inherited connective tissue disease characterized by progressive postnatal heterotopic ossification. The earliest pathological finding in FOP is perivascular lymphocytic infiltration into normal-appearing skeletal

muscle, followed by muscle-cell degeneration and highly vascular fibroproliferative soft tissue swelling. The fibroproliferative lesions evolve, through an endochondral process, into mature lamellar bone with marrow elements. Heterotopic ossifications are usually first detected around the spine and proximal extremities, then at multiple other places, which leads to dysfunction of articulations and often premature death.^{1,2} Although the precise genetic defects of this disease have not been identified, several studies have suggested that bone morphogenetic protein-4 (BMP4) may play a key pathophysiological role in this disease.³⁻⁵ BMPs, members of the TGF superfamily, were originally known as potent bone-inducing morphogens. Later studies showed that they play pivotal roles in many major common signaling networks, and regulate many processes in the embryo and adult. Disruption of these pathways has been implicated in a range of diseases ranging from developmental disorders to heritable cancer.⁶ Genetically manipulated mice that overexpress BMP4 under the control of several different promoters have been reported, but they fail to induce postnatal heterotopic ossification. For example, neither transgenic mice overexpressing BMP4 under control of a *keratin* promoter (*K14*),⁷ nor mice expressing BMP4 using an expression vector based on the *bovine cytokeratin IV* promoter develop heterotopic ossification.⁸ Transgenic mice overexpressing BMP4 under control of *surfactant protein-C* gene promoter die from abnormally formed lungs.⁹ Transgenic mice expressing *Bmp4* in cartilage under the control of the *Col11a2* promoter/enhancer sequences die at birth due to respiratory failure.¹⁰ *Msx1-Bmp4* transgenic mice overexpressing human BMP4 under control of mouse *Msx1* minimal promoter develop no visible abnormalities.¹¹ We report here a developmentally associated postnatal heterotopic os-

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sification mouse model: transgenic mice that overexpress BMP4 under the control of *neuron-specific enolase* (NSE) promoter. These transgenic mice develop severe postnatal heterotopic ossification, which closely recapitulates major FOP phenotypes. This study shows that overexpression of BMP4 alone is sufficient to trigger a cascade of events that leads to progressive postnatal heterotopic endochondral ossification *in vivo*. This animal model thus provides a unique opportunity to further study the role of the BMP signaling pathway in heterotopic ossification and to improve our understanding of the pathophysiologic aspects of FOP from the pre-clinical phases all the way through late stages of the disorder.

Materials and Methods

Transgenic Mice

Generation of NSE-BMP4 transgenic mice was described elsewhere.¹² The NSE-noggin transgene was constructed by cloning a fragment containing the full-length murine noggin cDNA downstream of the rat *neuron-specific enolase* (NSE) promoter and upstream of a SV40 polyadenylation signal. The NSE promoter construct contained the initial, noncoding exon of the rat NSE locus, which increases expression levels.¹² The transgene fragment was isolated and injected into CB6F₁ mouse embryos, and founder animals were identified by Southern blot analysis according to a standard protocol. Double-transgenic mice expressing both BMP4 and Noggin were obtained by crossing the NSE-BMP4 and NSE-Noggin transgenic mice, and screening the progeny for double transgenes. The primers used for routine PCR are:

For screening NSE-BMP: 5'-CACTGTGAGGAGTTTC-CATC-3' and 5'-GTGATGGACTAGTCTGGTGTGTC-3'.

For screening NSE-Noggin: 5'-AGGCCGGTGTGCG-TTGTG-3' and 5'-ATGTAAAGCATGTGCACCGA-3'.

For screening double-transgenic mice, both above-mentioned primer pairs were used.

Soft X-Ray Assessment

Animals were given whole body X-ray examination at 42kV, 25mA, 0.05 seconds (General Electric, Model 225, USA).

General Histology

Hematoxylin and Eosin Staining

Hematoxylin and eosin (H&E) staining was performed on fixed frozen tissue sections or decalcified plastic-embedded bone section, using Harris Modified Hematoxylin and Eosin Y Solution (Sigma, St. Louis, MO), according to manufacturer's instructions.

Alkaline Phosphatase (ALP) Staining

ALP staining of fixed frozen section of tissues was done as described by Sugiyama et al.¹³

Tartrate-Resistant Acid Phosphatase (TRAP) Staining

TRAP staining of fixed frozen section of tissues was done as described by Erlebacher et al.¹⁴

Alizarin Red and Alcian Blue Staining

Alizarin red and Alcian blue staining were done according to the protocols from The Internet Pathology Laboratory for Medical Education, Florida State University College of Medicine (<http://www-medlib.med.utah.edu/WebPath/webpath.html>).

Antibodies and Immunohistochemistry

Immunostaining for different markers was done according to standard protocols. Mouse anti-col I (SP1.D8), mouse anti-col II (CIIC1), mouse anti-bone sialoprotein (BSP II) (WVID1) and mouse anti-MSX (4G1) were obtained from the Developmental Studies Hybridoma Bank. Additional antibodies and their sources were as follows: mouse anti-BMP4 (Chemicon, Temecula, CA), goat anti-Runx2 (PEBP2 α A, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), rat anti-CD34, rat anti-CD45 and rat anti-Sca-1 (BD PharMingen, San Diego), lineage Cocktail 1 (Lin1: CD3, CD14, CD16, CD19, CD20, and CD56) (Becton Dickinson, San Jose), rabbit anti-human N-cadherin (IBL, Japan).

Polarized Light Microscopy Analysis

Frozen sections of heterotopic bone were analyzed under polarized light microscopy (Carl Zeiss Inc., Thornwood, NY), according to the manufacturer's instructions.

Skeleton Preparation

Mouse skeletons were prepared according to protocol from Behringer.¹⁵

Isolation of Peritoneal Cells and Peripheral-Blood Mononuclear Cells

Peritoneal cells were collected, following the protocol from Stall.¹⁶ Mononuclear cells were isolated by using density gradient separation kit (HISTOPAQUE-1077, Sigma) from mouse periphery blood, according the manufacturer's instruction.

Sex-Mismatched Cells Transplantation and Heterotopic Bones Transplantation

For sex-mismatched cells transplantation, peritoneal cells and mononuclear cells were isolated from 2-month-old male donor mice (NES-BMP4 transgenic mice or WT littermates), and cell numbers were counted. Five $\times 10^5$ peritoneal or mononuclear cells in phosphate-buffered saline (PBS) were injected subcutaneously to the inner side of female left hind limb of 2-month-old NES-BMP4

transgenic mice or WT littermates. For heterotopic bone transplantation, heterotopic bones were surgically removed from adult NES-BMP4 transgenic mice, and implanted subcutaneously in the back of 3- to 4-week-old NES-BMP4 transgenic mice or WT littermates.

Fluorescence in Situ Hybridization (FISH)

The donor cells were followed by dual color FISH (X-chromosome with FITC and Y-chromosome with Cy3), using STAR⁺FISH Mouse Whole Chromosome-Specific paint systems (Cambio, UK), following the manufacturer's instructions.

Results

Young NES-BMP4 Transgenic Mice Develop Severe Heterotopic Bone Formation, Apparently through Endochondral Ossification

Neuron-specific enolase (gamma enolase, NSE) is predominantly expressed by neurons and neuroendocrine cells, but much lower levels are also expressed by developing or injured striatal muscle cells,¹⁷ by activated T cells, and by activated macrophages.¹⁸ We initially constructed transgenic animals that overexpress either BMP4 (NSE-BMP4) or the BMP inhibitor noggin (NSE-noggin) under control of the NSE promoter to examine the role of BMP signaling in development of the brain.¹² However, serendipitously, the BMP4 transgenic mice also develop postnatally severe heterotopic bones. At approximately 2 months of age transgenic animals start to show a subtle visible phenotype of uneven gait, especially with the hind limbs. When picked up by the tail, the hind limbs of young NES-BMP4 transgenic mice appear stiff and cannot fully extend or move freely (Figure 1A). The first noticeable external phenotype of the NES-BMP4 transgenic mice is swelling of hind limb muscles with enlargement of the hind limb circumference (Figure 1B). At this stage, the only abnormality on physical examination besides the muscle swelling is slightly increased tissue turgor. Systematic histological and immunohistochemical analysis of tissue sections from hind limbs at this time found that the earliest histological changes were infiltration of CD45⁺ mononuclear cells into intramuscular regions (Figure 1C) with muscle fiber degradation and local proliferation of fibroblast-like cells, usually located subcutaneously (Figure 2A). However, infiltration of CD45⁺ cells in intramuscular regions was transient, and once the heterotopic ossification developed, the infiltration of CD45⁺ cells was usually no longer detectable. Further, although the muscle swelling and then heterotopic ossification were invariant features of the phenotype, infiltration of the CD45⁺ cells was not always observed.

The muscle swelling and local proliferation of fibroblast-like cells led directly to heterotopic endochondral ossification through several distinguishable phases. Phase I of heterotopic endochondral ossification started with local proliferation of fibroblast-like cells, which were

Figure 1. Early skeletal phenotypes of NES-BMP4 transgenic mice. **A:** The first noticeable phenotype of NES-BMP4 transgenic mice was stiffness of the hind limbs. **a:** When picked up by the tail, hind limbs of affected mouse showed sign of stiffness and cannot fully extend and move freely, **(b)** while WT littermate can fully extend their hind legs and move freely. **B:** The first gross phenotype of NES-BMP4 transgenic mice was enlargement of the hind limb circumference. **Lane 1,** WT littermates; **Lane 2,** NES-BMP4 transgenic mice before abnormality; and **Lane 3,** NES-BMP4 transgenic mice after abnormality. **C:** The first histological phenotypes were infiltration of CD45⁺ mononuclear cells and muscle degeneration. FITC was used to stain CD45, DAPI was used to stain nuclei, and autofluorescence in Cy3 channel was intentionally collected to show the basic structure of muscle fibers. **a:** A large amount of CD45⁺ mononuclear cells was detected. **Brace** indicated the region where muscle fibers degenerated. **b:** WT littermates showed no sign of CD45⁺ mononuclear cells infiltration or muscle degeneration.

first identified as a clump of cells usually located subcutaneously (Figure 2A). Histological and immunohistochemical studies found that most of these early proliferating cells were positive for alkaline phosphatase (ALP) (Figure 2Ab), Sca-1⁺ (Ly-6A/E⁺) (Figure 2Ac), CD34⁺ (not shown), BMP4 (Figure 2Ad), and Lin⁻ (Figure 2Ae). BMP4 expression was not evenly distributed, and the outer parts of the cell masses displayed much higher levels of BMP4 than the inner regions. This staining pattern suggested that these cells are mesenchymal stem cells (MSCs) (see <http://stemcells.nih.gov/stemcell/pdfs/appendix.pdf>). Phase II of heterotopic endochondral ossification was characterized by continued proliferation of the putative MSCs associated with the initiation of differentiation of some of the cells. The innermost cells lose CD34 (Figure 2Ba), BMP4 expression (Figure 2Bb), and Sca-1 (not shown) markers and became condensed. There was also obvious *de novo* angiogenesis initiated in the tissue adjacent to the dense cell mass (Figure 2B, c and d). The dense cell mass was still ALP positive (Figure 2Be) but was negative for Alcian blue at this phase (Figure 2Bf). Phase III of heterotopic endochondral ossification was characterized by the appearance of ALP⁺, Alcian blue⁺ and morphologically typical chondrocytes in the inner region (Figure 2C, a and b), while cells in the outermost surrounding region still expressed the CD34⁺ and Sca-1⁺ markers (data not shown). Phase IV started as early as 3 months of age at a time when swelling of the hind limbs increased greatly and the transgenic animals

Figure 2. Sequential events of heterotopic endochondral ossification in NSE-transgenic mice. **A:** Phase I of heterotopic endochondral ossification. **a:** The early histological finding is the local proliferation of fibroblast-like cells, usually located subcutaneously (shown) or between muscles (not shown). Note the fibroblast-like morphology (H&E staining). **b:** The early cells mass were positive for ALP (blue staining). **c:** Most of these cells bear MSCs markers Sca-1⁺ (FITC, shown) CD34⁺ (not shown). **d:** These cells expressed BMP4 (Cy3): the surrounding region had higher level of BMP4, while the inner region had lower level and **(e)** Lin (FITC) was negative. **B:** Phase II of heterotopic endochondral ossification. **a:** Inner cells lose CD34 (FITC) staining (shown) and Sca-1 staining (not shown) **b:** Early cell mass continually proliferated and expressed BMP4 (Cy3) (especially the surrounding region). **c:** Obvious *de novo* angiogenesis initiated in the connected tissue between the dense fibrotic region and skin. **Arrows** indicated blood vessels. **d:** H&E stain of dense fibrotic and its surrounding region. Note most of the cells in fibrotic region still kept fibroblast-like morphology at this stage. **Arrows** indicated blood vessels. **e:** Dense fibrotic region still kept ALP activities (surrounding region had stronger activities). **f:** Alcian blue staining was negative. **C:** Phase III of heterotopic endochondral ossification. **a:** At this stage, inner cell region already condensed and differentiated, and show typical chondrocyte morphology. **b:** ALP staining showed the inner cells lost ALP activities. **c:** Alcian blue staining showed the inner cells were positive. **D:** Phase IV of heterotopic endochondral ossification. Multi-focal central hypertrophic chondrocytes were surrounded by profound proliferative fibroblast-like cells. Around the marginal hypertrophic chondrocytes, nascent trabeculae-like calcium deposits appeared.

developed multiple small, hard subcutaneous nodules. Histochemical and immunocytochemical analysis of tissue sections from hind limbs at this stage revealed heterogeneous, subcutaneous populations of MSCs surrounding multi-focal central regions of hypertrophic chondrocytes (Figure 2D). Typical osteoblasts (ALP⁺, N-Cad⁺ CD45⁻) (Figure 3Aa and Bd) began to appear around the hypertrophic chondrocytes followed by nas-

cent trabeculae-like calcium deposits, which were confirmed by Alizarin red staining (Figure 3Ab and data not shown). Typical multinucleated tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts as well as occasional mononucleated TRAP⁺ cells appeared wherever the marrow space formed (Figure 3Ac). Later on, osteoblasts, calcium deposits, and osteoclasts replaced the central hypertrophic chondrocytes and mature tra-

Figure 3. A: Heterotopic bone showed characteristic structure of cancellous or woven bone. **a:** ALP staining of typical heterotopic bone. ALP activities (blue stain) were detected in trabeculae, especially on surfaces contacting with marrow space. **b:** Alizarin red staining of typical heterotopic bone. Note that trabeculae were strongly positive for the stain. **c:** TRAP staining of typical heterotopic bone. **Arrow** indicated typical multinucleated TRAP-positive cells and untypical mononucleus TRAP-positive osteoclasts cells located in marrow space, may or may not contact with trabeculae. **d:** Typical heterotopic bone under polar light. Lighter regions in trabeculae indicated regular arrangement of collagen fibers and darker regions indicated irregular arranged collagen fibers. **B:** Expression patterns of key transcription factors and markers implicated in osteogenesis; DAPI was used to stain nuclei in all case. **a:** Msx2 was detected in proliferative fibroblast-like mesenchymal cells. **b:** col II was detected mostly in typical chondrocytes or hypertrophic chondrocytes. **c:** col I was detected in presumably pre-osteoblasts or osteoblasts in newly formed trabeculae. **(d)** Double-staining of N-cad(Cy3) and CD45(FITC). Typical N-Cad⁺ CD45⁻ cells were detected in newly formed trabeculae. **e:** Runx-2 was detected in newly formed trabeculae. **f:** BSP was detected in newly formed trabeculae, matrix, or cytoplasmic.

beculae structures started to form, leading to radiopacities (Figure 4). Multi-focal calcification then presumably re-modeled and merged to form giant irregularly shaped heterotopic bones (Supplementary Figure 1). In the end, almost all the hypertrophic chondrocytes were replaced by a “spongy” 3-D trabecular latticework filled with marrow elements. Heterotopic bones showed characteristics of trabecular bones. The overall shape of the heterotopic bones was irregular, probably dependent on local anatomical environment. They looked red or pink to the naked eye (Supplementary Figure 1), because they were filled with red marrow. The microstructure of heterotopic bones, however, was an organized “spongy” 3-D trabecular latticework, which is formed by slender, irregular trabeculae, although the trabeculae of heterotopic bones found in NSE-BMP4 transgenic mice were much thinner (average $\sim 20 \mu\text{m}$ in diameter) than normal cancellous bones (100 to 200 μm in diameter). Within the cavities formed by irregular trabeculae was the marrow element, which accounts for most ($\sim 90\%$) of the total volume of heterotopic bones. There were many typical osteoblasts,

located in irregular trabeculae or the trabecular surfaces contacting the marrow space. There were also many typical osteoclasts (very large multinucleated TRAP-positive cells) as well as some atypical osteoclasts (smaller mononucleate TRAP-positive cell) located in the marrow space itself. Polarized light microscopy showed regular arrangement of collagen fibers in some regions of trabeculae (like lamellar bones) and irregular arranged collagen fibers in other regions (like woven bones) (Figure 3Ad).

We next sought to determine whether transcription factors that are known to regulate normal prenatal osteogenesis and skeletogenesis are also expressed during the process of postnatal heterotopic ossification. Msx2, a known target of BMP signaling, was highly expressed in the proliferating MSCs but not in other cell types (Figure 3Ba). Col II, a marker of pre-chondrocytes, proliferating chondrocytes, and hypertrophic chondrocytes, was expressed in an entirely different pattern in cells with the typical appearance of chondrocytes or hypertrophic chondrocytes in the transgenic animals (Figure 3Bb). Col I, a pre-osteoblast and osteoblast marker, was expressed

Figure 4. X-ray images of NSE-BMP4 transgenic mice and littermates. **A:** X-ray image at different time points of a typical NSE-BMP4 transgenic mouse and its littermate indicated the heterotopic ossification is progressive. **a:** Three-month-old NSE-BMP4 transgenic mouse and its littermate. **Arrows** indicated the defused radiopaque regions. **b:** Four-month-old NSE-BMP4 transgenic mouse and its littermate. **Arrows** indicated the radiopaque regions. Note that the radiopaque regions were expended. **c:** Five-month-old NSE-BMP4 transgenic mouse and its littermate. **Arrows** indicated the radiopaque regions. Note, not only the radiopaque regions were expended but also the typical kyphoscoliosis. **d:** Comparison of 8-month-old NSE-BMP4, NSE-Noggin, and NSE-BMP4, NSE-Noggin double-transgenic mouse. Note, only the NSE-BMP4 transgenic mouse had severe heterotopic ossification and kyphoscoliosis. **e:** One example of NSE-BMP4 mouse with fully developed heterotopic ossification. **B:** Spectrum of skeletal phenotypes in transgenic mice. Phenotypical comparison of six individual 5-month-old NSE-BMP4 transgenic mice. **Arrows** indicated the radiopaque regions. Note, some mice had severe and some mice had milder heterotopic ossification and kyphoscoliosis.

in newly formed trabeculae (Figure 3Bc), Runx2, an obligate transcriptional activator of osteoblast differentiation (also called *Osf2/Cbfa1*) was expressed in a subpopulation of cells where nascent trabeculae started to form (Figure 3Be). BSP, another pre-osteoblast and osteoblast marker, was also abundantly expressed in newly formed trabeculae, either in matrix or cytoplasm of presumably osteoblasts (Figure 3Bf). This analysis of postnatal heterotopic ossification in NSE-BMP4 transgenic mice is reminiscent of the patterns of mesenchymal cell condensation that occur during normal skeletal embryogenesis and suggests a common molecular basis for normal prenatal bone formation and postnatal heterotopic ossification.

Heterotopic Bone Formation Is Progressive and Follows a Predictable Pattern That Eventually Leads to Immobilization of the Mice

Serial physical examinations and X-ray images of different mice revealed that there was a relatively uniform phenotype with a stereotyped spreading pattern of heterotopic ossification. The process typically started in the hind limbs (Figure 4A), then spread dorsally to paravertebral regions and ventrally to the abdominal wall at about the same time, and finally spread to the anterior trunk, forelimbs, and skull. The entire process typically

took up to about 1 year (Figure 4A, a to e). Heterotopic ossification was primarily located in the subcutaneous connective tissue of the abdominal wall, proximal extremities, and the paravertebral region (Supplementary Figure 1). Osseous bridges developed in multiple locations, such as the pelvis and the jaws. In advanced cases, the osseous bridges developed even beyond existing joints; for example, huge masses of osseous bridges developed between fore and hind limbs in some mice, which almost totally immobilized the affected mice (Supplementary Movie). However, heterotopic ossification was never found in the tongue, diaphragm, and other viscera.

Interestingly, as in FOP patients, normal skeletogenesis was not significantly disturbed in NSE-BMP4 transgenic mice. Serial physical examinations and X-rays taken revealed no gross defects of skeletogenesis in young NSE-BMP4 transgenic mice when no obvious heterotopic bones had formed (Figure 4Aa). However, in older mice with fully developed heterotopic bones there were a variety of skeletal deformities including scoliosis, kyphosis, and kyphoscoliosis (Figure 4A, c to e and Figure 4B), which again resembles the phenotype found in FOP patients.

Postnatal Heterotopic Ossification Phenotype Is Rescued in NSE-BMP4 and NSE-Noggin Double-Transgenic Mice

The postnatal heterotopic ossification in two different lines of NSE-BMP4 transgenic mice suggested that BMP signaling plays a key role in heterotopic ossification. However, it was possible that the phenotype reflected a non-specific positional effect due to the sites of insertion of the transgene into DNA. To exclude this possibility, we crossed NSE-BMP4 transgenic mice with NSE-Noggin transgenic mice (under the same NSE promoter), screened and studied the phenotype of mice overexpressing both NSE-Noggin and NSE-BMP4. We reasoned that if overexpression of BMP4 specifically caused heterotopic ossification, co-expression of the inhibitor *in vivo* should rescue the phenotype. In fact, the phenotype of postnatal heterotopic ossification was completely rescued in these double-transgenic mice; no heterotopic ossification was detected in any of these double-transgenic mice ($n = 25$). This indicates that it is specifically overexpression of BMP4 that leads to heterotopic ossification.

Heterotopic Bones Lack Tumorigenic Activities

The progressively spreading nature of heterotopic bone formation raised the issue of whether this reflected tumorigenesis. To address this issue, heterotopic bone was surgically removed from NSE-BMP4 transgenic mice and implanted subcutaneously into the backs of 3- to 4-week-old NES-BMP transgenic mice or WT littermates with the same background, and the animals were observed for growth of the implants. Three- to 4-week-old mice were used since there is normally no endogenous heterotopic bone formation at this age. We did not find any growth of

Figure 5. A: Summary of tumorigenesis activities tests. The heterotopic bones were surgically removed from NSE-BMP4 transgenic mice and implanted subcutaneously in the back of NES-BMP transgenic mice or WT littermates. Cells extracted from heterotopic bones also subjected to soft agar colony analysis. There was no positive colony in soft agar analysis, and no growth of implants was observed in recipient mice either. **B:** Summary of sex-mismatched cell transplantation. Peritoneal cells and mononuclear cells were isolated from 2-month-old male donor NES-BMP transgenic mice (red lines) or male WT littermates (black lines), and were injected subcutaneously to the inner side of female left hind limb of 2-month-old NES-BMP4 transgenic mice or WT littermates. "no" indicated there was no heterotopic ossification within 2 weeks. "H.O." indicated there was heterotopic ossification within 2 weeks. Note that only female NES-BMP4 transgenic mice receiving the male NES-BMP4 donor cells developed heterotopic ossification. **C:** Dual-color FISH experiment to follow the donor cells in recipient mice. **a:** Y-chromosome (Cy3), **(b)** X-chromosome (FITC), **(c)** DAPI staining, **(d)** merge of **a-c** frozen section of tissue with heterotopic ossification from recipient mice. Note the typical chondrocytes or hypertrophic chondrocytes morphology and nuclei staining, and more importantly, many of typical chondrocytes or hypertrophic chondrocytes had both X and Y chromosome staining.

implants or heterotopic bone formation in any animal 2 months after the surgery. Soft agar clonal analysis also confirmed this finding; cells extracted from heterotopic bones did not form any visible colonies in soft agar whereas positive control cells (HEK 293T) did. (Data not shown). All these findings indicate the heterotopic bones have no demonstrable tumorigenic activities (Figure 5A).

Peritoneal Cells and Mononuclear Cells from NSE-BMP4 Can Contribute to Heterotopic Ossification

The heterotopic ossifications in the NSE-BMP4 transgenic mice seemed to follow the classic pathway of endochondral ossification. However, it was still unclear what cell type(s) initiated the process of heterotopic ossifications. We designed sex-mismatched cells transplantation experiments to address this question. Peritoneal cells and mononuclear cells were isolated from 2-month-old male NSE-BMP4 and WT littermates and injected subcutaneously into the hind limbs of 2-month-old female NSE-BMP4 and WT littermates. The donor cells were followed by dual-color FISH (Cambio, UK). Female NSE-BMP4 transgenic mice that received donor peritoneal and mononuclear cells from male NSE-BMP4 transgenic mice developed heterotopic ossifications within 2 weeks (Figure 5, B and C), whereas WT recipients and NSE-BMP4 animals that received WT tissue did not. Dual-color FISH on the frozen sections of heterotopic bones confirmed that many of the chondrocytes had both X and Y chromosome signals and were thus derived from the donor. This transplantation experiments suggested two interesting points: 1) since peritoneal and mononuclear cells are a different, mixed cell population, it is very likely that multiple cell sources may contribute to heterotopic ossifications; and 2) only cells derived from NSE-BMP4 mice and injected in NSE-BMP4 recipient mice contribute to heterotopic ossifications, which suggest that the genetic origins both of the donor cells and of the recipient mice are important in generation of heterotopic bones.

Discussion

Shafritz et al³ found that the ectopic osteogenesis of FOP is associated with increased levels of BMP4 in lymphocytes and Olmsted et al⁴ further found that the rate of BMP4 transcription is elevated in cells of FOP patients. This suggested that an inappropriate enhancement of the rate of BMP4 transcription may play a critical role in the molecular pathophysiology of FOP. Our animal model confirms that up-regulated BMP4 signaling can cause a FOP-like phenotype in mice that matches the anatomical, spatial, and temporal characteristics of the human disorder.

BMP4 has been implicated in various morphogenetic processes such as limb formation, neurogenesis, tooth formation, and other epithelial mesenchymal interactions in early embryos and skeletogenesis.^{7,10,19–21} Misexpression of BMP4 clearly induced *de novo* ectopic bone formation in our animal model, and the postnatal heterotopic ossification in these mice virtually copied the entire process of prenatal endochondral bone formation but in anatomically inappropriate places. Gene expression patterns of key transcription factors and markers of early normal prenatal osteogenesis and skeletogenesis also strongly suggested a common molecular basis for prenatal and postnatal osteogenesis. Thus, overexpression of BMP4 alone is sufficient to trigger a cascade of events

that leads to progressive postnatal heterotopic endochondral ossification *in vivo*, indicating that the *Bmp4* gene is able to trigger the entire series of events needed for bone formation, and overexpression of BMP4 probably also is a necessary factor to maintain and expand the heterotopic ossification.

What is the nature and source of the cells that initiate the process of ectopic bone formation in FOP? One recent report suggested a vascular origin of heterotopic ossification,²² but others have reported that muscle stem cells (satellite cells) may contribute to heterotopic ossification (Eleventh Annual Research Report of International FOP Association, <http://www.ifopa.org/pdf/Eleventh%20Annual%20Report.pdf>). Importantly, still others have reported that activation of inflammatory mast cells may play a role in the pathology of FOP lesions.²³ In fact, infiltration of CD45⁺ mononuclear cells was the earliest, although transient, cellular event that was detectable in our transgenic animals. Further, our sex-mismatched cell transplantation studies demonstrated that mononuclear cells or mixed populations of peritoneal cells could initiate the process of heterotopic ossification, which suggested that multiple sources could contribute to the heterotopic ossification in some conditions. It is still not clear what is (are) the real cell source(s) of *in vivo* heterotopic ossification. However the histological studies of NSE-BMP4 transgenic mice with early phenotypes demonstrated that the early proliferating cells all expressed MSC markers. Our hypothesis, based on our current animal studies and previous reports, is that the infiltration of tissue by BMP4-expressing mononuclear cells, perhaps in response to minor injuries, initiates the process by stimulating endogenous populations of MSCs. Some of the mononuclear cells themselves may be converted in this environment to cells that display the phenotype of MSCs although this has not been clearly demonstrated. MSCs then initiate the process of endochondral bone formation due to high local BMP signals. Furthermore, once stimulated, the MSCs themselves begin to express BMP4 (Figure 2) which keeps the process going in an autocrine fashion. Such a hypothesis would explain the results of the transplantation experiments that demonstrated that genetic origins of both the source of the donor cells and the recipient mice were critical for generating heterotopic bone formation. This would also explain why different authors studying different stages of FOP have implicated both mononuclear cells and stem cells in the process.

Mating of the NSE-BMP4 transgenic animals with mice that overexpress noggin under control of the same promoter completely rescued the animals from developing FOP. Further, Glaser et al²⁴ reported that administration of noggin can prevent the intramuscular calcification associated with intramuscular injection of BMP4. These observations strongly support the concept that noggin therapy may be an effective way of treating FOP. However systemic administration of noggin, or viral expression of noggin in all cells, might be expected to disrupt many of the normal cellular processes in which BMP signaling is important. The apparent critical role of infiltrating mononuclear cells in the pathophysiology of the disorder suggests that gene therapeutic overexpression of noggin

only in these cells might provide a viable approach. The availability of the animal model of FOP will now make it relatively easy to examine the efficacy of this and other possible methods of treatment.

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