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COMMENTARY

Encrypted Morphogens of Skeletogenesis

BIOLOGICAL ERRORS AND PHARMACOLOGIC POTENTIALS

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ABSTRACT. Bone morphogenetic proteins (BMPs) are members of a class of ancient, highly conserved signalling molecules that play major roles in embryonic axis determination, organ development, tissue repair, and regeneration throughout the animal kingdom. The bone morphogenetic proteins are potent developmental morphogens that act in a concentration-dependent manner to specify cell fates in developing and regenerating systems. Complementary DNAs have been cloned for approximately twenty BMPs, and recombinant proteins have been produced for many of these genes. Transgenic and naturally occurring animal models demonstrate a wide variety of potential functions for *BMP* genes during development and tissue regeneration, and a wide range of pharmacologic effects are predicted from knock-out or over-expression of the *BMP* genes. Fibrodysplasia ossificans progressiva (FOP), a rare and devastating genetic disease of ectopic osteogenesis in humans, is associated with over-expression of at least one of the *BMPs*. The BMPs, their transmembrane receptors, their intracellular signal transducers, and their secreted antagonists hold great promise as pharmacologic agents in modulating a vast array of developmental and regenerative pathways in human diseases. BIOCHEM PHARMACOL 55;4:373–382, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. bone morphogenetic protein (BMP); BMP type I and type II receptors; chordin and noggin; fibrodysplasia ossificans progressiva; heterotopic ossification; morphogen; SMAD proteins

FIBRODYSPLASIA OSSIFICANS PROGRESSIVA

Rare diseases often provoke important insights into more common disorders and provide a better understanding of normal physiology gone awry. FOP[‖] is an extremely rare, autosomal dominant disorder of connective tissue, with a prevalence of about one case per two million people [1, 2]. Those who have this disorder can be described as essentially forming two skeletons: a normotopic one during embryogenesis and a heterotopic one following birth. The postnatal heterotopic skeleton rigidly immobilizes the joints of the body, rendering movement impossible. Although FOP was first described more than 300 years ago, the genetic defect and pathophysiology remain unknown [2].

FOP is characterized by congenital malformation of the great toes (Fig. 1) and by progressive, disabling heterotopic osteogenesis in distinct anatomic patterns [3–7]. Spontaneous or trauma-induced exacerbations of FOP during childhood are characterized by massive painful soft tissue swelling (Fig. 1) [8]. Histopathologic studies of pre-osseous lesions reveal lymphocytic infiltration and muscle cell degeneration [9], followed by the appearance of highly vascular, fibroproliferative tissue and finally endochondral ossification with mature lamellar bone and marrow elements [10].

Heterotopic ossification in FOP begins in childhood and can be induced by minor trauma [11]. Severe scoliosis may develop due to asymmetric heterotopic ossification of paravertebral muscles [12]. By early adulthood, heterotopic ossification leads typically to ankylosis of all major joints of the axial and appendicular skeleton, rendering movement impossible [7, 8, 13] (Fig. 2). Most patients are relegated to a wheelchair by their early twenties and require life-long assistance in performing activities of daily living [4, 7]. Starvation and pneumonia are common causes of death [8]. Surgical trauma associated with resection of heterotopic bone leads to exacerbation of local ossification [8]. At present, there is no effective prevention or treatment.

IDENTIFICATION OF CANDIDATE GENES FOR FOP

The usual approach to identifying the genetic basis of a disease (by genetic linkage analysis and positional cloning) presently is impossible for FOP due to a low reproductive fitness that results in a small number of affected individuals and a lack of multi-generational families showing inheritance of the disease. The candidate gene approach is therefore being pursued as an alternative but indirect method of attempting to identify the mutated gene respon-

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‖ *Abbreviations:* BMP, bone morphogenetic protein; FOP, fibrodysplasia ossificans progressiva; TGF transforming growth factor; *dpp*, *decapentaplegic*; Osf, osteoblast-specific transcription factor; Cbfa, core binding factor alpha; CDMP, cartilage-derived morphogenetic protein; Gdf, growth differentiation factor; AJF, aggressive juvenile fibromatosis; and mRNA, messenger ribonucleic acid.

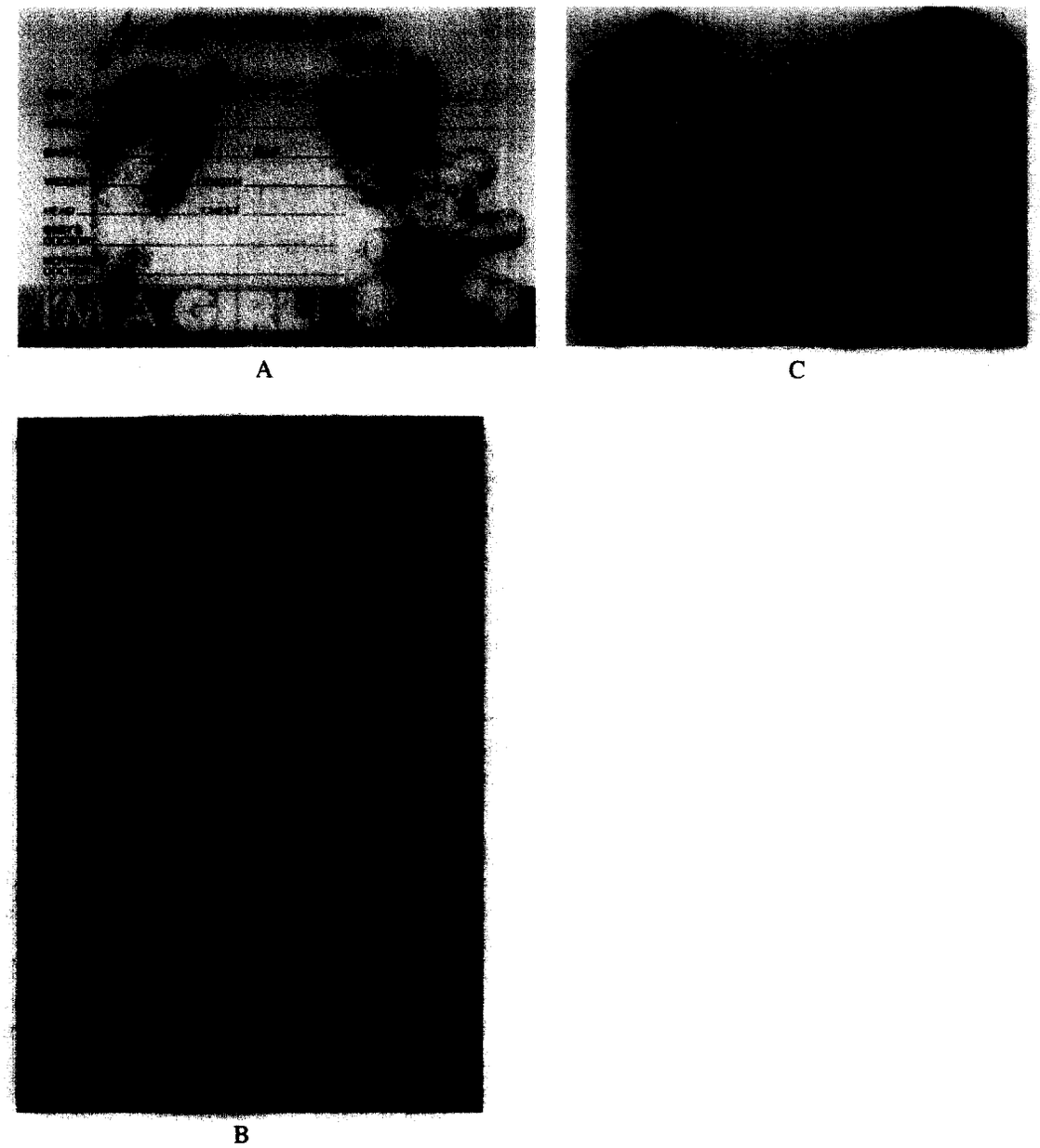

FIG. 1. Characteristic features of FOP in early childhood. The presence of short malformed great toes at birth (A and C) heralds the later spontaneous appearance of pre-osseous soft-tissue lesions on the neck and back (B) (arrow heads), and should provoke suspicion of FOP even before the transformation to heterotopic bone (arrows). An inspection of the toes will confirm the diagnosis and may alleviate the need for a lesional biopsy (trauma) that could exacerbate the condition. [Reprinted with permission from *J Bone Miner Res* 12: 855, 1997.] Copyright (1997) American Society for Bone & Mineral Research. [Ref. 3].

sible for FOP. In selecting candidate genes for FOP, the main diagnostic criteria (congenital malformations of the great toes with an anterior polarizing defect, heterotopic endochondral ossification, and temporal and spatial patterns of ectopic bone formation) must be considered. The candidate gene for FOP would need to be one that is functional during normal embryonic development (to account for the malformations of the great toe), and one that also could be activated post-natally to induce severe generalized heterotopic ossification in tendon, ligament, fascia, and skeletal muscle.

Genes that plausibly fit these criteria are the *BMP* genes and genes in the BMP pathway [14, 15]. BMP was the name initially given to a demineralized bone extract that had the ability to induce ectopic bone formation in an animal assay system [16].

BMPS AND BMP SIGNALLING PATHWAYS

Morphogens are secreted peptides that diffuse from their source (thus establishing gradients) and act on transmembrane receptors to specify cell fate in a concentration-dependent manner. Substantial data support the role of BMPs as morphogens in animal development [15, 17–23]. Many of the BMPs act as inducers of endochondral osteogenesis and fracture healing [14, 16–21, 24–28]. BMPs are

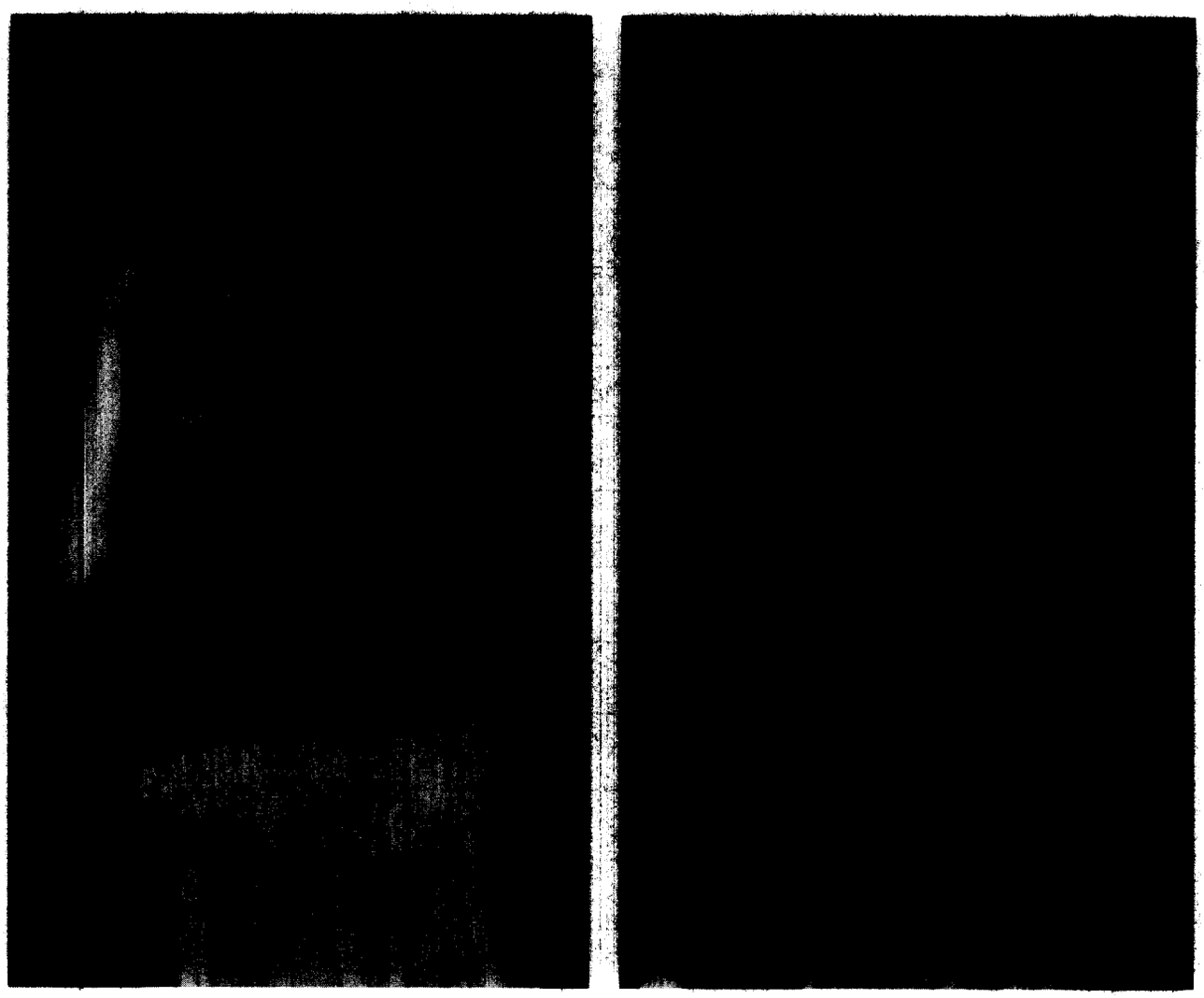

FIG. 2. Clinical photograph and skeleton of a man with FOP. The rigid posture noted in this 25-year-old man with FOP is due to ankylosis of the spine, shoulders, and elbows. Plates and ribbons of ectopic bone contour the skin over the back and arms (A), and can be visualized directly on the skeleton (B) (following death from pneumonia at age 40 years). Courtesy, Mütter Museum, College of Physicians of Philadelphia. [Reprinted with permission from *N Engl J Med* 335: 555–561, 1996; Fig. 1.] Copyright (1996) Massachusetts Medical Society. All rights reserved. [Ref. 13].

unique in their ability to induce the complete cellular program of endochondral osteogenesis at heterotopic sites *in vivo* [14, 15, 29–31]. Although their family name implies a predominant role in skeletogenesis, the BMP functions include somite development, limb patterning, neural crest lineage determination, and development of the kidneys, teeth, lung, heart, liver, gut, testes, skin, and skeleton [19, 32–38] (Table 1).

Based on similarity of protein structure, the BMPs are part of the larger TGF-β superfamily of peptides [15]. BMPs are the most phylogenetically conserved members of the TGF-β superfamily and are ubiquitous regulators of animal development [15]. Among the BMPs, BMP-4 plays a vital and seminal role in the induction of all mesoderm [21].

Interestingly, the genes with the highest degree of similarity to members of the mammalian BMP family have been found in the fruit fly, *Drosophila melanogaster* [22, 52]. The *BMP-2* and *BMP-4* genes, which produce proteins that are about 90% similar to each other, are homologous to the *Drosophila dpp* gene. The DPP protein shows ~75% amino acid identity to BMP-2 and BMP-4 in the mature carboxy-terminal region of these proteins [22, 52]. As secreted peptides, they act as dorsalizing gradient morphogens in dipteran development, and as ventralizing gradient morphogens in vertebrate development [15, 53]. The dipteran and vertebrate BMP-4 homologs are able to substitute functionally for each other *in vivo* in genetic complementation studies [22, 54].

Hox genes [18], *hedgehog* genes [55], fibroblast growth factor genes [56], and the Ras/Raf/AP-1 pathway genes are putatively involved in BMP-4 signalling [57]. The developmental action of the BMPs can be modulated *in vivo* by the secreted peptides chordin/sog and noggin [56, 58–65], which have a high binding affinity for the BMPs and competitively inhibit the binding of BMP to its transmembrane receptors [58]. In vertebrate neurogenesis, the BMP

TABLE 1. Established tissue expression for vertebrate bone morphogenetic proteins

BMP	Skl	Ten	Tth	Skn	Msc	Bnm	Hrt	Lng	Liv	Fat	Gut	Kid	Pan	Cns	Eye	Prs	Ovy	Tst	References
BMP-2/dpp	+		+	+			+	+				+					+		[37, 39, 40]
BMP-4/dpp	+		+	+	+		+	+			+	+		+					[17, 39]
BMP-5	+							+	+			+							[39, 41]
BMP-6/Vgr-1	+							+				+							[39, 42, 43]
BMP-7/OP1	+		+	+				+				+			+				[35, 36, 39, 44]
BMP-8a/OP2																		+	[39, 41]
BMP-8b																		+	[32, 39]
CDMP-1/Gdf-5	+																		[26, 39]
BMP-13/Gdf-6	+																		[45]
BMP-12/Gdf-7		+																	[4
BMP-3/osteogenin	+							+			+	+					+		[39, 41]
Gdf-10/BMP-3b	+				+		+	+					+	+					47]
BMP-9									+										[4
Gdf-1														+					[49]
Gdf-3						+				+									[50]
Gdf-8					+														[51]
Gdf-9																	+		[50]

Expression includes both embryonic and post-natal expression. See Ref. 39 for a general review. Abbreviations: Skl, skeleton (bone or cartilage); Ten, tendon/ligament; Tth, teeth; Skn, skin/hair follicle; Msc, muscle/somites; Bnm, bone marrow/spleen/thymus; Hrt, heart; Lng, lung; Liv, liver; Fat, adipose tissue; Gut, gut/intestine/; Kid, kidney; Pan, pancreas; Cns, brain/CNS; Prs, prostate, Ovy, ovary; Tst, testes; OP, osteogenic protein; and Vgr, Vg (vegetal) related.

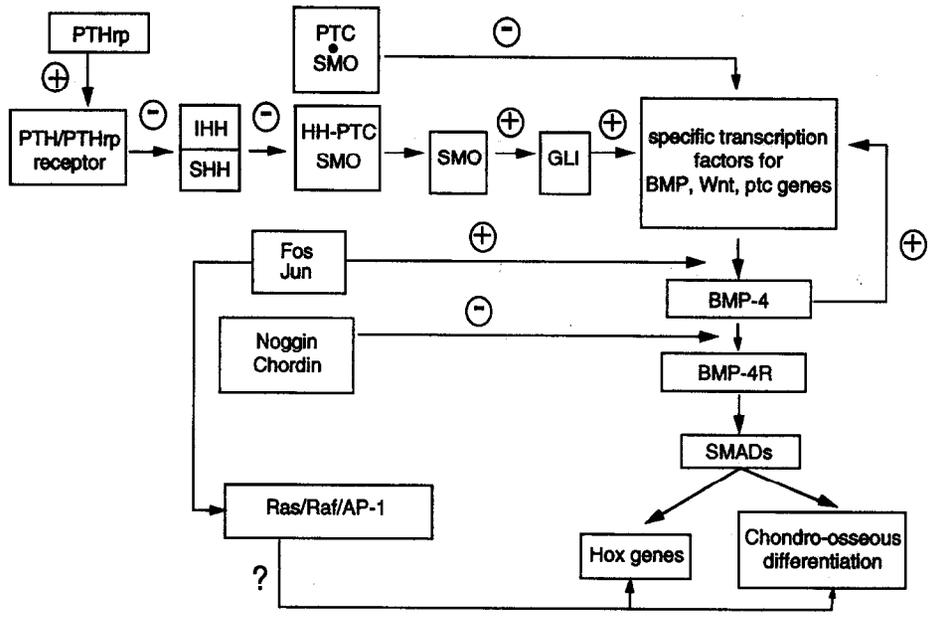

FIG. 3. Putative BMP-4 signalling pathways. This composite schematic diagram depicts experimentally determined features of BMP signalling that have been identified in numerous *in vitro* and *in vivo* model systems including *Drosophila*, *Xenopus*, chicken, mouse, and human. Each component of this pathway may not be active in every model system or in every cell type within a model system. Key: (+) indicates an activating pathway; and (−) indicates an inhibitory pathway. Abbreviations: PTHrp, parathyroid hormone related protein; PTH, parathyroid hormone; IHH, Indian hedgehog; SHH, sonic hedgehog; HH, headgehog (either IHH or SHH); PTC or ptc, patched; SMO, smoothened; GLI, glioblastoma-derived oncogene family; BMP, bone morphogenetic protein; Wnt, vertebrate wingless family; BMP-4R, receptors for BMP-4; Hox, vertebrate homeobox family; Fos and Jun, members of the AP-1 family of transcription factors.

antagonists noggin and chordin (secreted by the Spemann organizer during early gastrulation in *Xenopus*) inhibit BMP activity, thereby allowing a default neural cell fate to develop rather than an epidermal one [56, 59–65]. Later in neurogenesis, BMPs are expressed selectively in the developing nervous system and play a major role in specifying the fate maps of neural crest cells [66]. Upstream regulators of BMPs have been identified and include the hedgehog family of signalling molecules (sonic hedgehog and Indian hedgehog), which bind to the patched receptor on the cell surface to derepress the activity of the transmembrane protein smoothened. This activity leads to expression of downstream transcription factors and ultimately to induction of *BMP* expression [67–69] (Fig. 3).

Few downstream targets of BMP activity have been identified. *Osf2/Cbfa1* is a recently cloned and obligate transcriptional activator of osteoblast differentiation regulated by BMP activity. Osf2/Cbfa1 protein binds to the promoter to regulate the expression of numerous genes expressed in osteoblasts. The spurious expression of *Osf2/Cbfa1* in pluripotent mesenchymal cells and in mouse skin fibroblasts induces the mature osteoblast-specific phenotype. *Osf2/Cbfa1* is positively regulated by BMP-7 and BMP-2 and inhibited by 1,25-dihydroxyvitamin D [70, 71]. Homozygous knockout of the *Osf2/Cbfa1* gene in mice results in complete lack of bone formation in both the endochondral and intramembranous pathways due to a failure of osteoblastic differentiation and a failure of hematopoietic development [71]. Mice heterozygous for the *Osf2/Cbfa1* deletion exhibit skeletal abnormalities characteristic of the human skeletal disorder cleidocranial dysostosis [72]. Mutations in *Osf2/Cbfa1* cause cleidocranial dysplasia in humans, and heterozygous loss of function is sufficient to produce the phenotype [73]. Conversely, *BMP* over-expression leads plausibly to over-expression of *Osf2/Cbfa1* and a robust osteogenic phenotype.

BMPs exert their biological effects via heteromeric (type I and type II) serine-threonine kinase transmembrane receptors [74–82]. Despite their ability to bind type I and type II receptors separately, BMPs require the steric interaction of these two receptors for optimal binding and signal transduction [80]. The intracellular effects of the BMPs are transduced by the cytoplasmic SMAD proteins, a class of transcription co-factors that are translocated to the nucleus following BMP-mediated cytoplasmic phosphorylation [77, 83, 84]. Little is known about the immediate downstream molecular targets of BMPs in vertebrate development [85].

Null mutations in the genes of two members of the BMP family have been identified in mammals. Each of these mutations results in highly restricted skeletal abnormalities during embryogenesis. Homozygous deletions of the *BMP-5* gene have been identified in the *short ear* mouse. These mutations lead to malformations of the axial skeleton and also result in abnormal fracture repair [25]. Homozygous mutations in *CDMP-1*, the human homologue of mouse *Gdf-5*, lead to Hunter–Thompson acromesomelic chondrodysplasia, a recessive disorder with skeletal abnormalities restricted to the limbs and synovial joints [86]. Homozygous

mutations of *Gdf-5*, a member of the BMP family, have been identified in the *brachypodism* mouse and result in malformations of the appendicular skeleton [26].

The absence of BMP-4 in a transgenic knockout mouse (genetically engineered to have no functional *BMP-4* genes) is lethal in early embryogenesis, showing little or no mesodermal differentiation and no hematopoiesis [20, 21]. BMP-4 has also been implicated in patterning of the developing mouse limb. In addition, over-expression of *BMP-4* in the chick embryonic limb bud has been associated with ectopic osteogenesis [87]. Targeted disruption of BMP-4 signalling in the same model leads to defects in apoptosis and failure to form the interdigital spaces [82]. These highly informative *BMP* mutations provide evidence for a direct role for at least some of the BMPs in embryonic and post-natal bone formation [15]. Thus, BMPs play critical roles in early embryogenesis and in skeletal formation, important criteria in considering them as plausible candidate genes for FOP.

INVESTIGATING THE CELLULAR AND MOLECULAR BASIS OF FOP

Drosophila genetics, mouse genetics, and developmental biology have provided us with several clues toward understanding BMP function and toward selecting the *BMPs* as reasonable candidate genes for FOP [52]. Studies in our laboratory have examined the expression of many of the *BMPs* in cells from FOP patients.

Early FOP lesions are histologically indistinguishable from AJF lesions; however, these two disorders can be distinguished by immunohistochemistry with BMP-2/4 antibodies [88]. Cells from AJF lesions (which do not progress to form bone) show no binding of the BMP-2/4 antibody. FOP lesional cells and tissue bind the BMP-2/4 antibody, indicating the presence of BMP proteins within very early FOP lesions [88]. The antibody used for these experiments cannot distinguish between BMP-2 and BMP-4. However, the activity of these two *BMP* genes can be distinguished by examining specific mRNA expression [13].

Northern analysis and ribonuclease protection assays were performed to specifically examine the expression of BMP-2 and BMP-4 mRNAs in cells from FOP patients [13]. Cells derived from a pre-osseous FOP lesion and from immortalized lymphoblastoid cell lines established from FOP patients show increased expression of BMP-4 but not BMP-2 compared with controls [13]. Correlation of BMP-4 expression with FOP was also observed in a family showing inheritance of FOP: The affected father and three affected children expressed BMP-4, while the unaffected mother did not. Further studies have verified that BMP-4 protein is synthesized in cells from patients who have FOP [13].

We have proposed that over-expression of a *BMP* gene may be involved in the gain-of-function leading to heterotopic ossification in patients with FOP [52]. Since the half-life of BMP is extremely brief (only a few minutes), it is unlikely that osteogenic-inducing concentrations of BMP-4 could be achieved at sites of osteogenesis unless the morphogen is delivered to those sites by circulating cells or manufactured at those sites by mesenchymal cells.

We have proposed that lymphocytes capable of expressing BMP-4 circulate in the peripheral blood of patients with FOP, and are recruited to connective tissue sites after soft-tissue injury [89, 90]. Type IV collagen, a primary constituent of the basement membrane of endothelial cells and muscle cells, avidly binds BMP-4 [91], resulting in increased local concentrations. At high concentrations, BMP-4 acts as a morphogen [14–16, 92] capable of up-regulating its own mesenchymal expression [25] and leading to the development of pre-osseous fibroproliferative lesions [14]. The appearance of lymphocytes in the perivascular space of the earliest detectable FOP lesions [9] provides support for the hypothesis that lymphocytes and perivascular cells may be involved in the induction of osteogenesis [93, 94].

Given the evidence of BMP-4 over-expression associated with heterotopic ossification in some patients with FOP, we are currently pursuing several directions of study to understand the exact involvement of BMP-4 in the pathophysiology of FOP. Recent results have indicated that the increased levels of BMP-4 mRNA in FOP cells are due to an increased rate of transcription of the *BMP-4* gene [95]. The increased activation of *BMP-4* in FOP cells may be due to a mutation within the *BMP-4* gene itself or, more likely, to a mutation in another genetic locus that causes over-expression of *BMP-4* in the cells of FOP patients. To begin to examine these questions, we are characterizing the structure and function of the human *BMP-4* gene in order to understand how the *BMP-4* gene is regulated. To determine whether circulating cells can be a plausible delivery vehicle for biologically significant quantities of BMPs, we are making transgenic animals that over-express BMPs in lymphocytes and other mononuclear cells of hematopoietic origin.

RELATIONSHIP OF BMP-4 SIGNALLING PATHWAYS TO FOP?

At the present time, a direct genetic link of FOP to the *BMP-4* gene has not been proven and remains very circumstantial. The genetic mutation(s) in FOP could plausibly reside anywhere in the BMP-4 signalling pathway, or in other signalling pathways that have effects on the level of *BMP-4* expression (Fig. 3). Our research, in the broadest sense, involves an analysis of the genetic and molecular pathways that are ectopically activated in patients who have FOP. We believe that this knowledge will also lead to more effective treatments of congenital skeletal disorders and problems in fracture healing.

PHARMACOLOGIC POTENTIAL OF THE BMPS AND RELATED MOLECULES

The BMP family of morphogens and their developmental antagonists provide a stunning array of recombinant pep-

tides with dramatic potential for pharmacologic use in humans. Studies have already commenced using recombinant BMPs for a wide array of orthopaedic conditions including augmentation of spinal fusions, promotion of osteogenic ingrowth into total joint arthroplasties, bone reconstruction following surgery for skeletal neoplasia, treatment of fracture non-unions, promotion of healing following osteonecrosis, and bone augmentation for dental and craniofacial reconstruction [96–105]. Many studies suggest that specific recombinant BMPs may have potential use in the regeneration of tendons and ligaments, and in treating various disorders of the urogenital and muscular systems [35, 36, 51, 106].

Creative uses of BMP and BMP-pathway technology will likely have important applications in the inhibition of heterotopic ossification in diseases such as FOP and even in more common forms of clinically disabling heterotopic ossification as encountered following closed head trauma, spinal cord injury, or total hip arthroplasty. Soluble BMP receptors, dominant negative receptors, and recombinant BMP antagonists such as chordin and noggin may be promising in binding and physiologically inactivating BMP where it is not needed or wanted [107].

The hope for an effective treatment for FOP has certainly been boosted by the recent discoveries of *BMP-4* over-expression in some patients who have the condition. Investigations are underway to identify the cause of this error in over-expression, which may be in the regulatory regions of the *BMP-4* gene or in some other gene whose product regulates *BMP-4* expression. Already, however, the findings have heightened hopes for an eventual cure for FOP. "With so much being discovered about how the BMPs act," says Brigid Hogan, a developmental geneticist at Vanderbilt University in Nashville, Tennessee, "it might be possible to develop drugs that would block some part of the BMP-4 pathway—and therefore prevent the progression of what is a horrible, nightmare disease" [108].

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