

## Fibromodulin is Expressed by Both Chondrocytes and Osteoblasts During Fetal Bone Development

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**Abstract** Fibromodulin, a keratan-sulfate proteoglycan, was first isolated in articular cartilage and tendons. We have identified fibromodulin as a gene regulated during BMP-2-induced differentiation of a mouse prechondroblastic cell line. Because expression of fibromodulin during endochondral bone formation has not been studied, we examined whether selected cells of the chondrocytic and osteoblastic lineage expressed fibromodulin. Fibromodulin mRNA was detected in conditionally immortalized murine bone marrow stromal cells, osteoblasts, and growth plate chondrocytes, as well as in primary murine calvarial osteoblasts. We, therefore, investigated the temporo-spatial expression of fibromodulin in vivo during endochondral bone formation by in situ hybridization. Fibromodulin was first detected at 15.5 days post coitus (dpc) in the perichondrium and proliferating chondrocytes. Fibromodulin mRNA was also detected at 15.5 dpc in the bone collar and periosteum. At later time points fibromodulin was expressed in the primary spongiosa and the endosteum. To determine whether fibromodulin was expressed during intramembranous bone formation as well, in situ hybridization was performed on calvariae. Fibromodulin mRNA was present in calvarial osteoblasts from 15.5 dpc. These results demonstrate that fibromodulin is developmentally expressed in cartilage and bone cells during endochondral and intramembranous ossification. These findings suggest that this extracellular matrix protein plays a role in both endochondral and intramembranous bone formation. *J. Cell. Biochem.* 82: 46–57, 2001. © 2001 Wiley-Liss, Inc.

**Key words:** fibromodulin; development; endochondral bone formation; intramembranous bone formation; matrix proteins

The skeleton is a specialized class of connective tissue that provides mechanical and protective properties, as well as supporting metabolic functions, of all vertebrates. The principal cells of the skeleton, the osteoblasts and chondroblasts, synthesize the extracellular matrix (ECM), which is critical for the normal structure and function of skeletal tissue [Marks and Hermey, 1996]. Although the ECM is composed primarily of collagens, 10% of matrix proteins are non-collagenous [Marks and Hermey, 1996]. It is well known that non-collagenous proteins interact with collagens and with

other matrix proteins to maintain skeletal integrity [Robey et al., 1992; Robey and Boskey, 1995; Robey, 1996]. Among these non-collagenous proteins, is a family of small leucine-rich repeat proteins (SLRP), which play an important role in ECM organization in many connective tissues [Robey, 1996; Iozzo, 1998]. To date, members of this family include biglycan, decorin, fibromodulin, lumican, chondroadherin, proteoglycan-Lb, osteoglycin, osteoadherin, and keratocan [Iozzo, 1998]. Overall these proteins are characterized by a core protein containing leucine-rich repeats, flanked on either side by cysteine residues [Iozzo, 1998; Matsushima et al., 2000]. Fibromodulin, lumican, chondroadherin, osteoglycin, osteoadherin, and keratocan carry keratan sulfate glycosaminoglycan side chains at their N-termini, while proteoglycan-Lb carries dermatan sulfate glycosaminoglycan side chains and the other members of the family are chondroitin sulfate proteoglycans [Robey, 1996; Iozzo, 1998]. Decorin and biglycan, are expressed both in cartilage and bone [Bianco et al., 1990; Robey,

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1996; Iozzo, 1998; Matsushima et al., 2000], whereas osteoglycin, and osteoadherin have been shown to be rather specific for bone [Bentz et al., 1989; Wendel et al., 1998]. Fibromodulin has been detected in embryonic sclerotomes at 9.5 days post coitus (dpc) [Wilda et al., 2000], however, at later stages of development its expression was thought to be restricted primarily to articular cartilage and tendon [Nurminskya and Birk, 1996; Wendel et al., 1998; Murphy et al., 1999]. The other members of the SLRP family are expressed in several connective tissues including cartilage, but not in bone [Heinegard et al., 1986; Larsson et al., 1991; Shinomaura and Kimata, 1992; Hedlund et al., 1994; Corpuz et al., 1996; Yng et al., 1997].

Fibromodulin, like decorin and biglycan, binds to TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3 and is thought to modulate the activity of these growth factors [Yamaguchi et al., 1990; Hildebrand et al., 1994; Takeuchi et al., 1994]. Members of the TGF $\beta$  superfamily have also been shown to regulate the expression and the synthesis of decorin and biglycan [Westergren-Thorsson et al., 1991; Takeuchi et al., 1993; Lecanda et al., 1997; Takagi et al., 1999; Yamada et al., 1999]. However, the expression and synthesis of fibromodulin is not regulated by TGF- $\beta$  [Takagi et al., 1999] and the effect of BMP-2 on fibromodulin expression has not been studied.

Using differential display PCR, we identified fibromodulin as a gene regulated as a prechondroblastic cell line, MLB13MYC clone 17, [Rosen et al., 1994] acquires markers of the osteoblast phenotype in response to BMP-2. As reported by Rosen et al. [1994] upon 24 h of BMP-2 treatment, these cells rapidly lose chondrocytic markers and express osteoblastic markers including type I collagen and osteocalcin mRNA. Since the BMP-2-induced expression of osteoblastic markers by MLB13MYC clone 17 cells parallels molecular events seen during endochondral bone formation *in vivo*, these cells are a suitable model for studying genes expressed and regulated during endochondral bone formation.

Because fibromodulin was regulated during MLB13MYC clone 17 cell acquisition of the osteoblastic phenotype, we examined fibromodulin expression in cells of both the chondrocytic and osteoblastic lineage *in vitro* and investigated its temporospatial expression *in vivo* during endochondral and intramembranous bone formation.

## METHODS

### Reagents

Tissue culture media, FBS, trypsin-EDTA, and penicillin-streptomycin were obtained from Gibco/BRL (Grand Island, NY). Molecular biology reagents, PGEM-T easy vector system and enzymes were purchased from Promega (Norwalk, CT). Trizol was obtained from Sigma. Radioisotopes were purchased from Dupont New England Nuclear (Boston, MA). The random primer labeling kit (Megaprime DNA labelling systems) was from Amersham (Piscataway, NJ). QuikHyb Solution was purchased from Gibco/BRL (Grand Island, NY). Nylon membranes were obtained from Biotrans ICN (Aurora, OH). RNAimage<sup>®</sup> and Message-Clean<sup>®</sup> kits were purchased from GenHunter Corp (Nashville, TN). NTB2 Photoemulsion was purchased from Kodak (Rochester, NY). The MLB13MYC clone 17 cell line and BMP-2 were kindly provided by Dr. V. Rosen (Genetics Institute). Conditionally immortalized murine bone marrow stromal cells, murine osteoblasts, and murine chondrocytes were kindly provided by Dr. F.R. Bringhurst (Massachusetts General Hospital, Boston, MA).

### Cell Culture

The prechondroblastic cell line, MLB13MYC clone 17, was isolated and characterized by myc immortalization of embryonic Day 13 mouse limb bud cells [Rosen et al., 1994]. MLB13MYC clone 17 cells were maintained as previously reported [Rosen et al., 1994] in DMEM supplemented with 10% heat inactivated (HI)-FCS and 1% penicillin/streptomycin. At confluence, the cells were treated with 0, 200, and 500 ng/ml BMP-2 in DMEM containing 1% HI-FCS and 1% penicillin/streptomycin for 8, 16, 24, and 96 h. Untreated cells were used as negative controls for each time point. Fresh medium with and without BMP-2 was added every 24 h. Conditionally immortalized murine bone marrow stromal cells, osteoblasts, and growth plate chondrocytes were cultured as previously reported [Divieti et al., 1998; Liu et al., 1998]. Conditionally immortalized murine growth plate chondrocytes were treated with or without 100 ng/ml of BMP-2 for 96 h [MacLean et al., 1999]. Primary calvarial osteoblasts from 18.5 dpc embryos were obtained as previously described [Divieti et al., 1998] and were

cultured in  $\alpha$ MEM supplemented with 10% HI-FCS and 1% penicillin/streptomycin.

#### Differential Display (dd) PCR

Total RNA, isolated from MLB13MYC clone 17 cells treated with BMP-2 (0, 200, and 500 ng/ml) for 8, 16, or 24 h, was first treated with RNase-free DNase I to eliminate contaminating chromosomal DNA. Two independent ddPCR reactions were performed according to the manufacturer's instructions. The radioactive PCR products were resolved on denaturing 6% polyacrylamide sequencing gels. After overnight autoradiography, differentially expressed bands were identified by comparison of ddPCR products representing mRNA species from BMP-2 treated and untreated cells. ddPCR products that were reproducibly differentially expressed were reamplified according to the manufacturer's instructions, subcloned into PGEM-T easy and subjected to DNA sequence analysis.

#### Northern Blot Analysis

Total RNA was isolated from MLB13MYC clone 17 cells treated with BMP-2 (0, 200, and 500 ng/ml) for 8, 16, or 24 h. Ten microgram of total RNA were resolved on a 1% agarose/formaldehyde gel and transferred to a nylon membrane by capillary blotting. Probes were radiolabeled with [ $\alpha$ - $^{32}$ P] dATP to a specific activity of  $\geq 10^8$  cpm/ $\mu$ g DNA. Control hybridization with an 18S rRNA antisense oligonucleotide verified that equal amounts of RNA were loaded.

#### In Situ Hybridization

Hind limbs and calvariae from 14.5 to 18.5 dpc embryos and from neonatal mice were fixed in 10% buffered formalin followed by dehydration and paraffin embedding. Tissue blocks were cut into 6  $\mu$ m sections, deparaffinized, and rehydrated. After rehydration the slides were fixed in 4% paraformaldehyde, digested with 10  $\mu$ g/ml of proteinase K and incubated with 0.2 N HCl for 10 min. After 10 min in a solution of 0.1 M triethanolamine and acetic anhydride, the slides were dehydrated through graded ethanol incubations and air-dried. Riboprobes were radiolabeled with  $^{35}$ S-UTP to a specific activity of at least  $10^8$  cpm/ $\mu$ g of riboprobe template. Hybridization was performed at 55  $^{\circ}$ C for 20 h in 50% formamide, 10 mM Tris-HCl pH 7.6, 200  $\mu$ g/ml tRNA, 1X Denhardt's solution, 10%

dextran sulfate, 600 mM NaCl, 0.25% SDS, and 50 mM DTT. Sequential washes with graded stringency were performed, following which slides were dipped in photoemulsion and exposed at 4 $^{\circ}$ C. After developing, the slides were counter stained with hematoxylin and eosin to identify the cellular source of the signal. Control experiments using fibromodulin sense riboprobes were performed to insure specificity of the signal.

#### RESULTS

Total RNA, isolated from MLB13MYC clone 17 cells treated with 0, 200, or 500 ng/ml of BMP-2 for 8, 16, and 24 h, was used for ddPCR experiments. As previously reported, in response to BMP-2 stimulation, these cells acquire markers of the osteoblast lineage including expression of type I collagen and osteocalcin [Rosen et al., 1994]. After overnight autoradiography bands, representing differentially expressed mRNAs, were identified. One of the differentially expressed PCR products, induced within 16 h of BMP-2 treatment (Fig. 1, arrow), was a 210 base pair product whose sequence was 99% identical to the 3' untranslated region of mouse fibromodulin mRNA (bases 2502–2712) (Genebank X94998).

Northern analyses, using total RNA isolated from MLB13MYC clone 17 cells probed with the

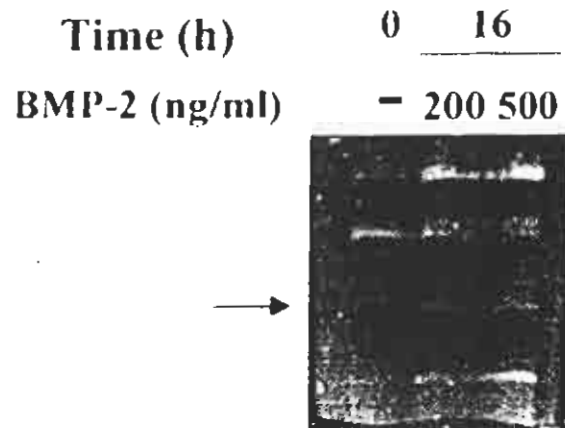


Fig. 1. Differential display PCR products from MLB13MYC clone 17 cells. Cells were treated for 16 h with 0, 200, or 500 ng/ml of BMP 2. RT-PCR reactions were performed using 0.1  $\mu$ g/ml total RNA. The resultant first strand of cDNA was amplified using an oligo dT primer anchored with a unique 3' base (AAGCTT) and a degenerate 5' oligonucleotide primer in the presence of 2  $\mu$ Ci [ $\alpha$ - $^{32}$ P] dATP for 40 cycles. The radioactive PCR products were resolved on a 6% polyacrylamide sequencing gel. Arrow indicates the candidate cDNA that appears to be differentially expressed.

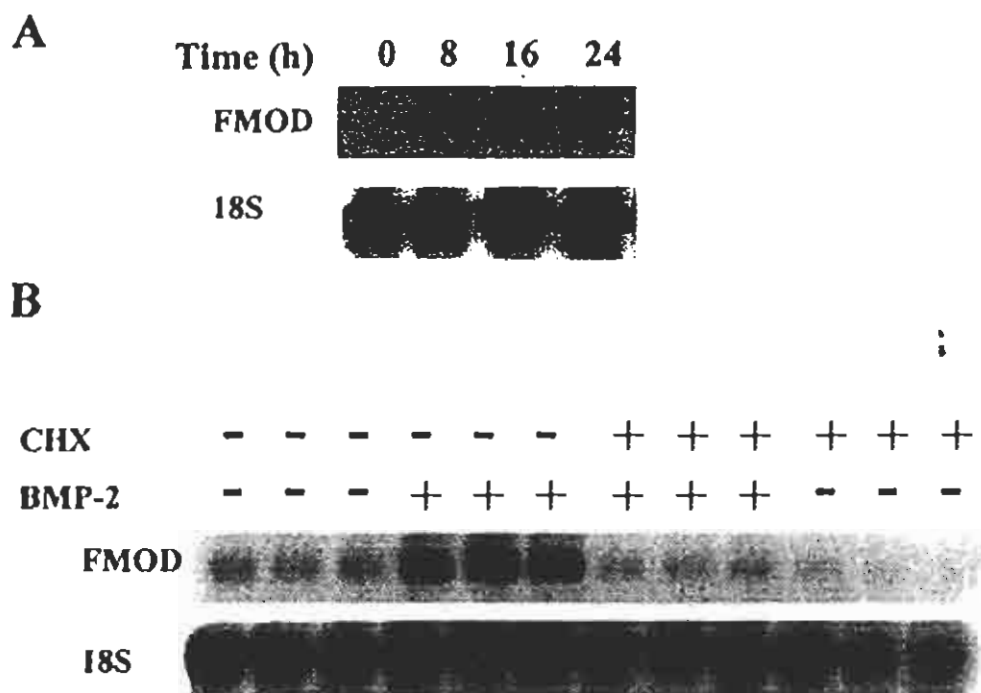


Fig. 2. Northern analysis. Total RNA isolated from MLB13MYC clone 17 cells treated or not with 200 ng/ml of BMP-2, was transferred to nylon membranes and hybridized to an  $\alpha^{32}\text{P}$ -labeled cDNA probe for fibromodulin (2.7 kb). **A**: Time course for fibromodulin expression (FMOD). **B**: Effect of cycloheximide (CHX) on the induction of fibromodulin expres-

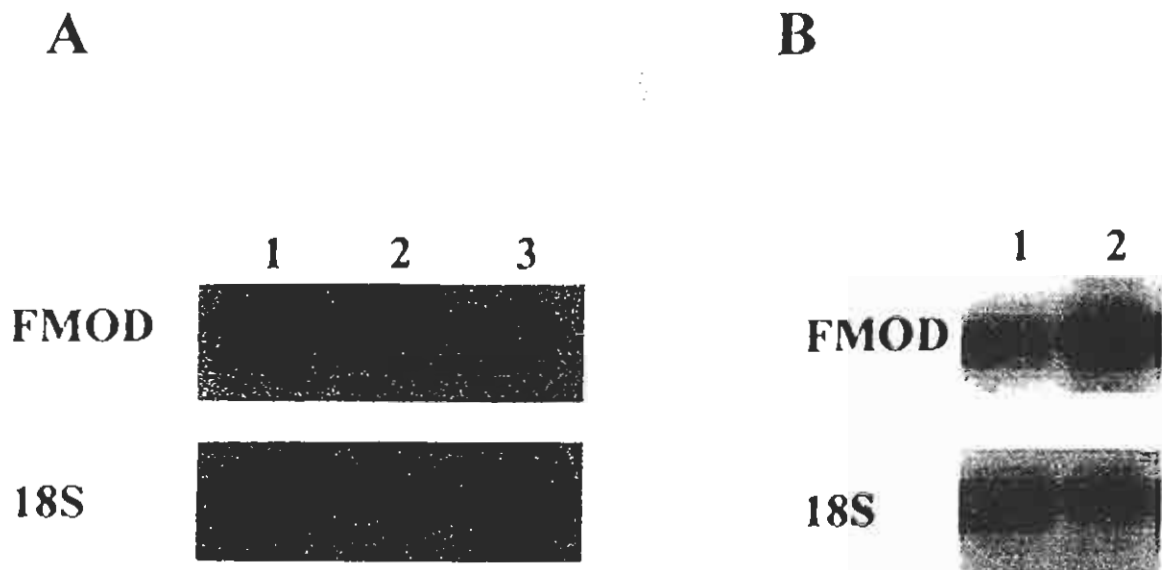
sion. CHX (1  $\mu\text{g}/\text{ml}$ ) was added 2 h before BMP-2 (200 ng/ml) and continued for the duration of BMP-2 treatment (24 h). The experiments were carried out at least three times, and a representative blot is shown. Control hybridization with an 18S rRNA probe verified the amount of RNA loaded.

cDNA for fibromodulin, detected a single mRNA transcript of 2.7 kb (Fig. 2A). Fibromodulin mRNA expression was induced 2.4-fold and 5-fold at 16 and 24 h of BMP-2 treatment, respectively. Since fibromodulin expression was induced early in MLB13MYC clone 17 cells, before classical markers of osteoblast differentiation [Rosen et al., 1994], cycloheximide (CHX) experiments were performed to address whether new protein synthesis was required for fibromodulin induction. Previous studies in our laboratory have demonstrated that 1  $\mu\text{g}/\text{ml}$  of CHX inhibits greater than 90% of new protein synthesis in the MLB13MYC clone 17 cells, as assessed by  $^{35}\text{S}$  methionine incorporation [Kearns and Demay, 2000]. Therefore, for these experiments CHX, at this dose, was added 2 h before BMP-2 (200 ng/ml) and for the duration of BMP-2 treatment (24 h). As shown in Figure 2B, CHX inhibited induction of fibromodulin mRNA, suggesting that new protein synthesis is required for this response.

Since MLB13MYC clone 17 cells have features of prechondroblastic cells and, upon BMP-2 treatment acquire features of osteoblasts, we

next investigated whether selected chondrocyte and osteoblast cell lines express fibromodulin. Fibromodulin mRNA was detected in conditionally immortalized murine osteoblasts and bone marrow stromal cells isolated from 18.5 dpc embryos, as well as in primary calvarial osteoblasts from 18.5 dpc embryos (Fig. 3A). Fibromodulin expression was also detected in conditionally immortalized murine growth plate chondrocytes, and 96 h of BMP-2 treatment (100 ng/ml) increased fibromodulin mRNA levels by 2.4-fold in these cells as well (Fig. 3B).

Since fibromodulin was expressed in vitro by cells of the chondrocytic and osteoblastic lineage, we performed in situ hybridization to determine whether osteoblasts and growth plate chondrocytes express fibromodulin in vivo. In tibiae of newborn mice, fibromodulin mRNA was detected in articular and proliferating chondrocytes (Fig. 4A and B). As shown in Figure 4B, fibromodulin expression decreased toward the distal part of the growth plate, such that prehypertrophic and hypertrophic chondrocytes were negative for fibromodulin (Fig. 4A



**Fig. 3.** Northern analysis. Total RNA, isolated from cells of the osteoblast and chondroblast lineage, was transferred to nylon membranes and hybridized with an  $\alpha^{32}\text{P}$ -labeled cDNA probe for fibromodulin (FMOD). **A:** Lane 1: conditionally immortalized primary osteoblasts from 18.5 dpc embryos; Lane 2: conditionally immortalized murine marrow stromal cells;

Lane 3: primary calvarial osteoblasts from 18.5 dpc embryos. **B:** Conditionally immortalized murine growth plate chondrocytes treated (lane 2) and untreated (lane 1) with 200 ng/ml BMP-2 for 96 h. The experiments were carried out twice, and a representative blot is shown. Control hybridization with an 18S rRNA probe verified the amount of RNA loaded.

and B). Fibromodulin was also detected in the osteoblasts of the primary spongiosa, the periosteum, and the endosteum (Fig. 4A and B). As shown in Figure 4C no signal was detected when sense probe was used, confirming specificity of the signal.

The novel observation that fibromodulin was expressed in growth plate chondrocytes and osteoblasts *in vitro* and *in vivo* led us to examine the temporal expression of fibromodulin during endochondral bone formation (embryonic Days 14.5–18.5 pc). As shown in Figure 5, fibromodulin was first detected at Day 15.5 pc. At this stage, fibromodulin was expressed in the proliferating chondrocytes, perichondrium, and bone collar (Fig. 5B and G). At 16.5 dpc (Fig. 5C and H) fibromodulin was also seen in the articular chondrocytes. As shown in Figure 5D, E, I, and J, at 17.5 and 18.5 dpc the expression of fibromodulin is highest in the periarticular and proliferating chondrocytes and absent in the hypertrophic chondrocytes. No signal was detected when fibromodulin sense probe was used, confirming specificity of the signal (data not shown).

To determine the cell types expressing fibromodulin, relative to that of specific cartilage markers, *in situ* hybridization was performed in

parallel sections of tibiae and femora from 14.5 to 18.5 dpc embryos. Although fibromodulin was not detected at 14.5 dpc, aggrecan, type II collagen, and matrix Gla protein (specific cartilage markers) were abundantly expressed in the proliferative chondrocytes (data not shown). Indian Hedgehog and patched, genes which play a critical role in the regulation of endochondral bone formation [Hooper and Scott, 1989; Bitgood and McMahon, 1995; Vortkamp et al., 1996], were also detected at 14.5 dpc (data not shown). At Days 16.5 and 18.5 pc, aggrecan was expressed throughout the growth plate (Fig. 6E and J), whereas fibromodulin expression was limited to periarticular chondrocytes and chondrocytes of the proliferating zone, overlapping the expression of patched (Fig. 6B, C, G, and H). As chondrocytes differentiate along the growth plate, fibromodulin expression decreased and both prehypertrophic and hypertrophic chondrocytes, which express Indian Hedgehog (Fig. 6D and I) and BMP-2 (data not shown), were negative for fibromodulin at all time points.

We next examined the temporo-spatial expression of fibromodulin relative to that of specific osteoblastic markers including Cbfa1, type I collagen, and osteocalcin [Stein and Lian,

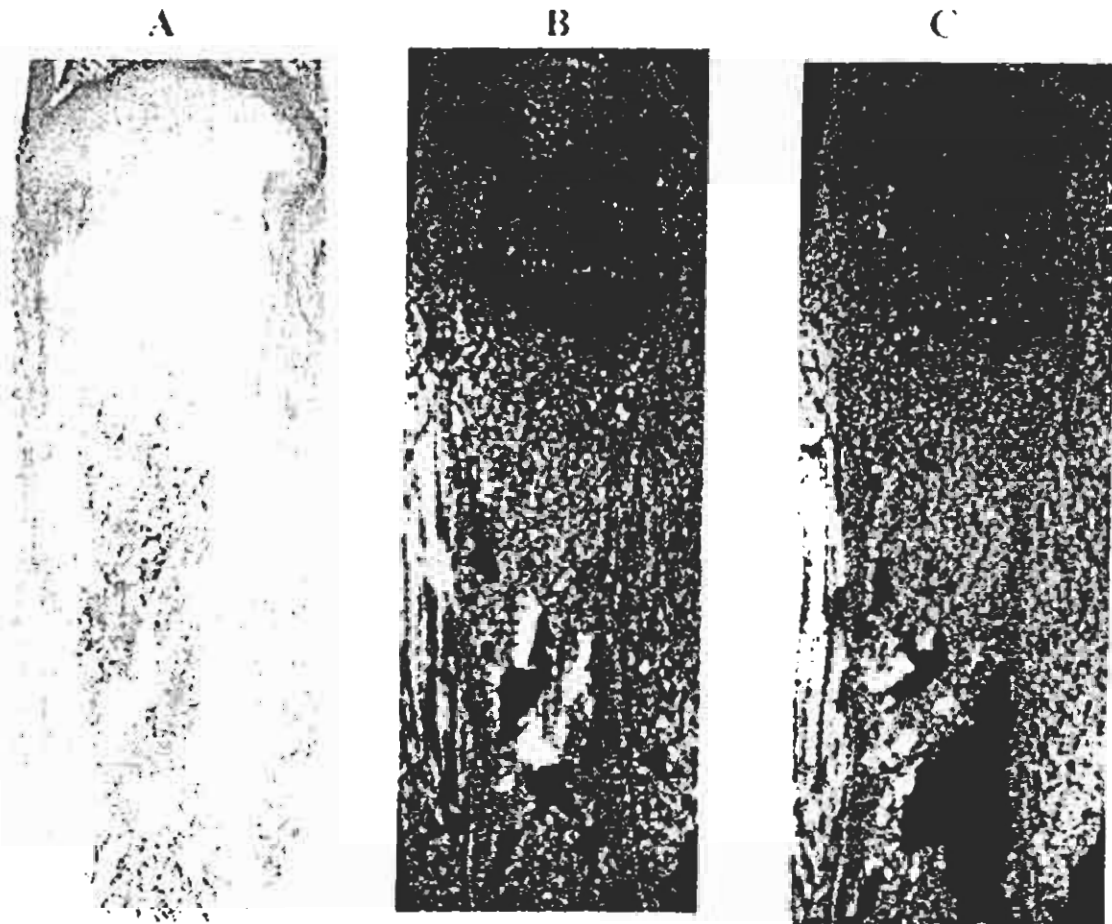


Fig. 4. Fibromodulin mRNA expression in newborn mice tibiae. Sense and antisense fibromodulin riboprobes were radiolabeled with  $^{35}\text{S}$ -UTP to a specific activity of at least  $10^6$  cpm/ $\mu\text{g}$  of template. Light field (A) photograph of a section of a newborn mouse tibia; dark field photographs of serial sections probed with antisense (B) or sense (C) fibromodulin riboprobes.

Hybridization was performed on sections from two tibiae and representative photographs are shown. Arrow indicates fibromodulin expression in the endosteum and arrowhead indicates fibromodulin expression in the periosteum. Magnification = 10X.

1993; Ducy and Karsenty, 1996; Ducy et al., 1997; Komori et al., 1997, Otto et al., 1997] in parallel sections of tibiae and femora from 14.5 to 18.5 dpc embryos. Like fibromodulin, type I collagen, a gene expressed by preosteoblastic cells [Stein and Lian, 1993], was first detected at 15.5 dpc (data not shown), whereas *Cbfa1*, a transcription factor involved in commitment and differentiation of cells to the osteoblast lineage [Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997], was present in areas of mesenchymal condensation (data not shown) at 14.5 dpc. At day 16.5 pc fibromodulin (Fig. 7B), expression overlapped that of *Cbfa1* in the primary spongiosa and periosteum (Fig. 7C) and that of type I collagen in the bone collar, primary spongiosa, and periosteum (Fig. 7D). These findings suggest that fibromodulin, like

*Cbfa1* and type I collagen, is expressed by early osteoblastic precursors including preosteoblasts. As shown in Figure 7E, osteocalcin, a gene expressed only in mature osteoblasts [Stein and Lian, 1993], was undetectable at 16.5 dpc. At Day 18.5 pc fibromodulin expression persisted in the bone collar, the primary spongiosa, the endosteum, and the periosteum (Fig. 7G) once again overlapping the expression of *Cbfa1* in the primary spongiosa and the periosteum (Fig. 7H), of type I collagen in the bone collar, the primary spongiosa and the periosteum (Fig. 7D) and of osteocalcin in the periosteum (Fig. 7J). Overall these data demonstrate that fibromodulin is expressed in early osteoblast precursors, including preosteoblasts as well as in terminally differentiated osteoblasts.

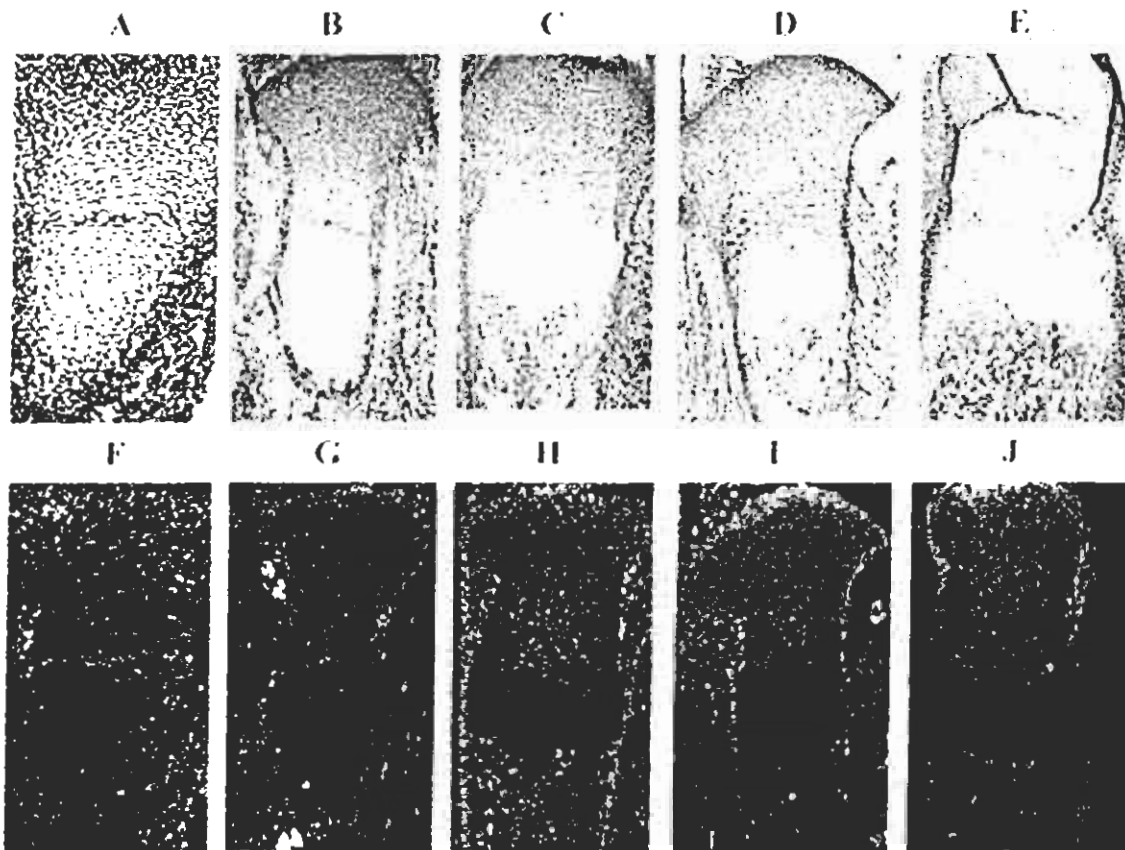


Fig. 5. Fibromodulin expression during endochondral bone formation. Fibromodulin antisense riboprobe was radiolabeled with  $^{35}\text{S}$ -UTP to a specific activity of at least  $10^8$  cpm/ $\mu\text{g}$  of riboprobe template. Light field photographs (A-E), dark-field (F-J) photographs of serial sections of developing mice proximal tibiae (14.5-18.5 dpc embryos) probed with a

fibromodulin antisense riboprobe. A and F: 14.5 dpc; B and G: 15.5 dpc; C and H: 16.5 dpc; D and I: 17.5 dpc; E and J: 18.5 dpc. Hybridization was performed on sections from at least three limbs and representative sections are shown. Magnification = 10X.

Since fibromodulin mRNA was expressed during endochondral bone formation, we next investigated whether fibromodulin was also expressed during intramembranous bone formation. For this purpose, we examined calvariae in which ossification occurs via direct differentiation of mesenchymal precursor cells into osteoblasts, and we compared fibromodulin expression to that of type I collagen. Type I collagen was detected at 14.5 dpc in calvarial osteoblasts (data not shown) whereas fibromodulin mRNA was first detected at 15.5 dpc (Fig. 8B). As shown in Figure 8B and E fibromodulin expression overlapped that of type I collagen both at 15.5 (Fig. 8C) and 18.5 dpc (Fig. 8F).

#### DISCUSSION

Our investigations demonstrate that fibromodulin is expressed in cells of both the chondrocytic and osteoblastic lineage, and that

its expression is regulated by BMP-2 *in vitro*. *In vivo*, fibromodulin mRNA is expressed during both endochondral and intramembranous bone formation. We speculate that the effect of BMP-2 on fibromodulin gene expression *in vivo* is likely to be similar to that seen *in vitro*. The temporal expression of BMP-2 and fibromodulin in the developing growth plate supports this contention, as does the observation that the cells that express fibromodulin are adjacent to those that express BMP-2. Similar spatial relationships are seen between other regulatory molecules and their targets or receptors in the developing growth plate, such as that between Indian hedgehog and its receptor, patched, as well as one of its target genes, PTHrP [Vortkamp et al., 1996].

Although we have shown that fibromodulin is expressed in the developing growth plate and in maturing osteoblasts, these novel observations



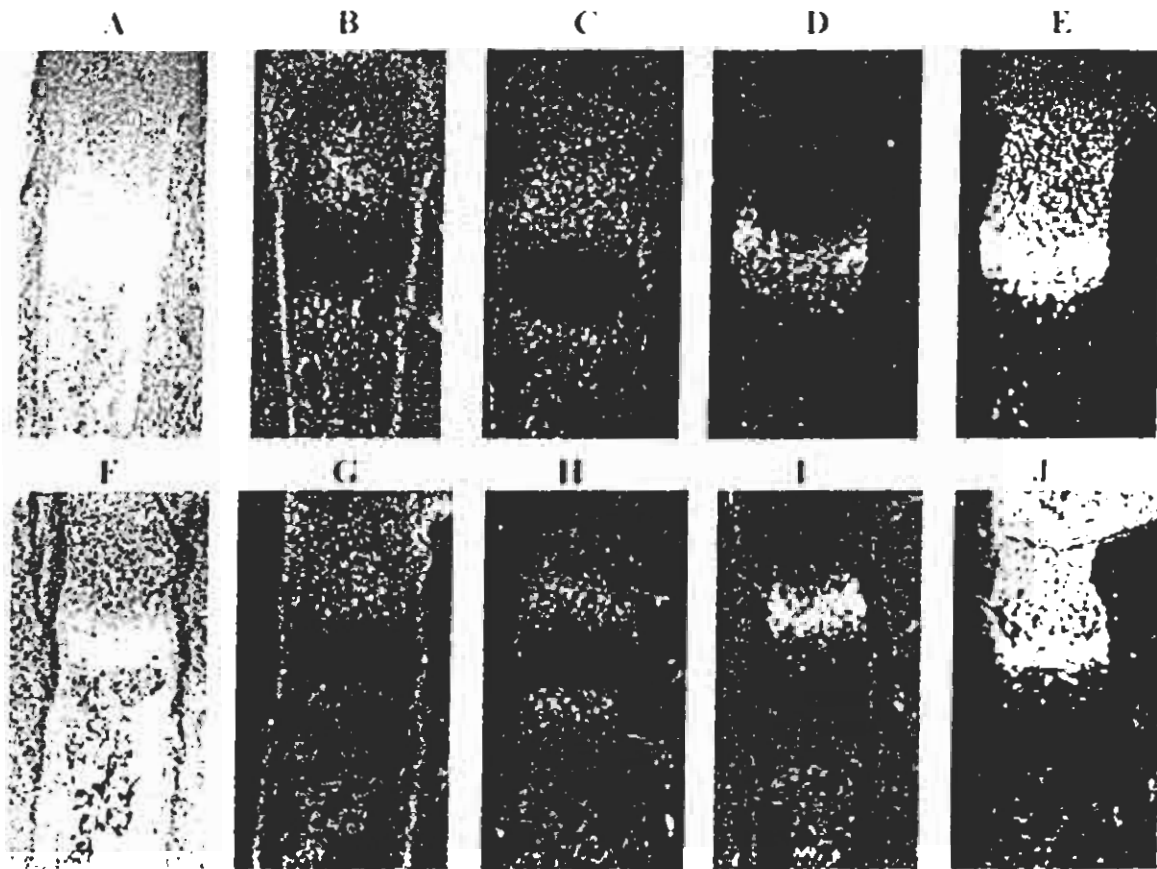


Fig. 6. In situ hybridization using chondrocyte markers. Fibromodulin, perlecan, aggrecan, and type II collagen antisense riboprobes were radiolabeled with  $^{35}$ S-UTP to a specific activity of at least  $10^6$  cpm/ug of template. Light field photographs of proximal tibiae of 16.5 dpc (A) and femora of 18.5 dpc embryos (F). Dark field photographs of proximal tibiae of 16.5 dpc (B-E)

and femora of 18.5 dpc embryos (G-J), fibromodulin (B and G), perlecan (C and H), Indian Hedgehog (I and J), and aggrecan (E and F) expression. Hybridization was performed on sections from at least three limbs and representative sections are shown. Magnification = 10X.

provide little insight into the role of this protein during endochondral bone formation. Recently, fibromodulin null mice have been generated and their phenotype analyzed [Svensson et al., 1999]. Although no overt skeletal phenotype was observed, ultrastructural and biomechanical analyses revealed abnormalities in collagen fibril organization in tendons [Svensson et al., 1999]. The lack of apparent skeletal phenotype in the absence of a gene that is developmentally expressed in chondrocytes and osteoblasts may be explained by one of several hypotheses. One possible explanation is that, since fibromodulin is expressed early, a delay in endochondral bone formation may be present during embryogenesis, but not evident in the adult skeleton due to the marked skeletal remodeling that occurs during growth and development. Alternatively,

a potentially more plausible explanation is that fibromodulin is functionally redundant in endochondral and intramembranous bone formation. In this scenario, other members of the family of the SLRPs would compensate for the lack of fibromodulin during skeletal development, and substitute for fibromodulin in the molecular interactions required for matrix organization and formation. In the tendons of fibromodulin null mice, lumican synthesis is increased suggesting that lumican can assume some of fibromodulin's roles in collagen fibril organization [Svensson et al., 1999]. However, mechanical and ultrastructural abnormalities are still detected in these tendons. Perhaps, in an analogous fashion, lumican or another family member is upregulated in the skeleton of fibromodulin null mice and subtle detect-



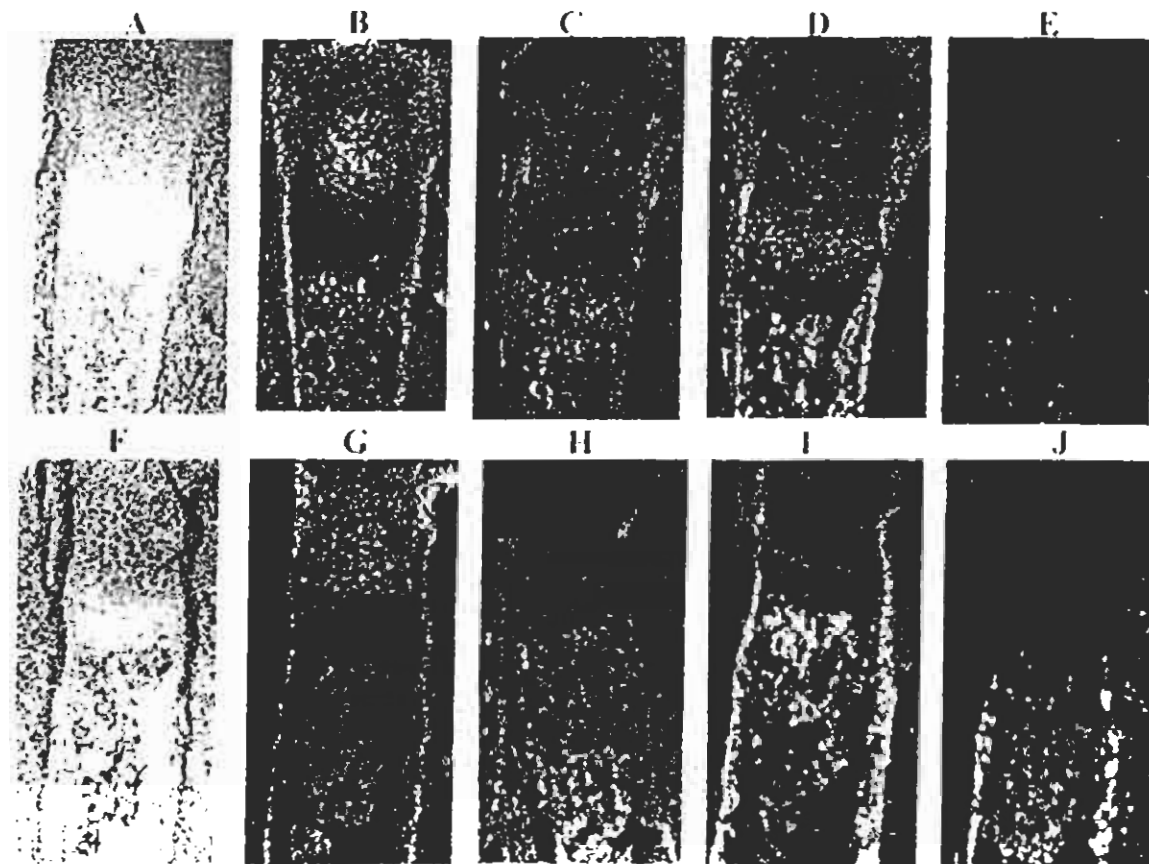


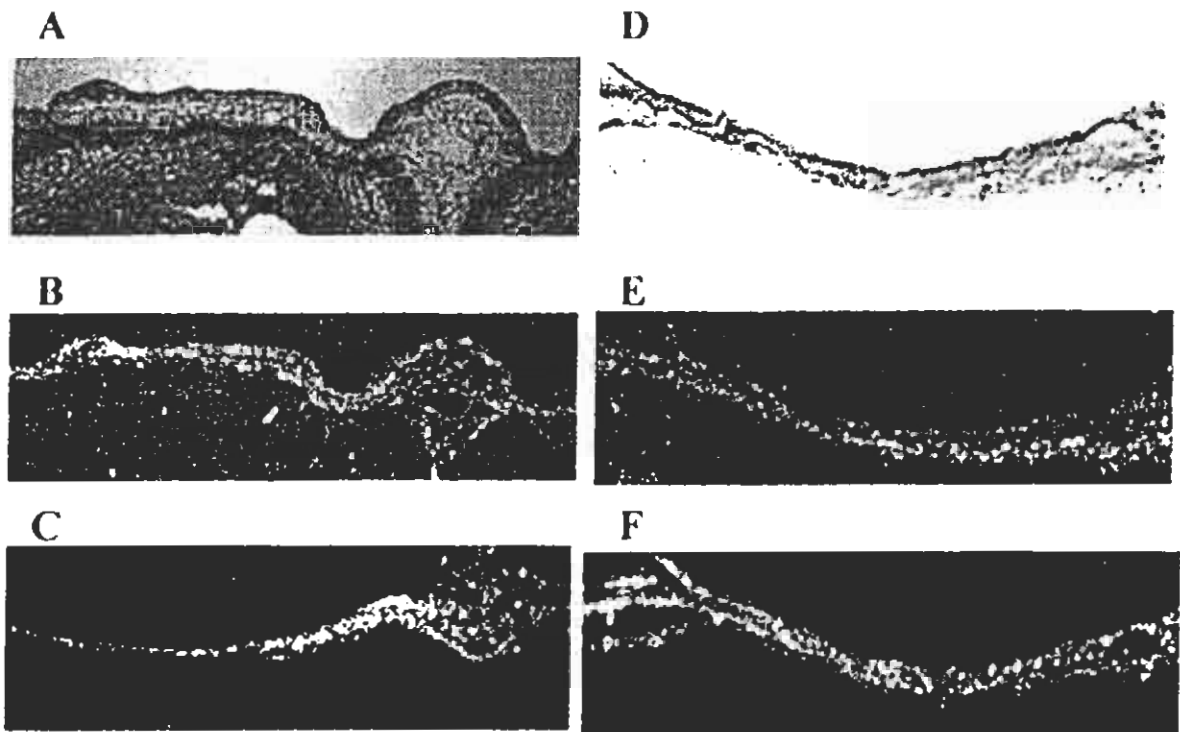
Fig. 7. In situ hybridization using osteoblast markers. Fibromodulin (A), type I collagen, and osteocalcin antisense riboprobes were radiolabeled with  $^{35}\text{S}$ -UTP to a specific activity of at least  $10^5$  cpm/ $\mu\text{g}$  of template. Light field photographs of proximal tibiae of 16.5 dpc (A) and femora of 18.5 dpc embryos (F). Dark field photographs of proximal tibiae of 16.5 dpc

and femora of 18.5 dpc embryos (G-J). Fibromodulin, B and C, (Dla1) (C and E). Type I collagen (D and H) and osteocalcin (F and J) expression. Hybridization was performed on sections from at least three limbs and representative sections are shown. Magnification = 10X.

could be unmasked in the skeleton of mature knockout mice by formal biomechanical analyses. The observation that, although both lunican and fibromodulin are expressed in the skeleton, targeted disruption of either of them fails to reveal a skeletal phenotype [Chakravarti et al., 1998; Svensson et al., 1999], suggests that these peptides can substitute for one another in skeletal matrix organization. In a similar fashion, although decorin is abundantly expressed in cartilage and bone, targeted deletion of its gene results in skin fragility due to lack of collagen fibril organization [Danielson et al., 1997]. Radiographic studies in two month old decorin knockout mice fail to demonstrate any abnormality in cartilage or bone [Danielson et al., 1997]. Like decorin, biglycan is also expressed in cartilage and bone, however,

biglycan mice do demonstrate a skeletal phenotype characterized by reduced bone formation, resulting in an osteoporosis-like phenotype by six months of age [Xu et al., 1998].

It is possible, therefore, that several members of the family of SLRPs can substitute for one another in the maintenance of skeletal homeostasis. It has been reported that decorin, biglycan, and fibromodulin can all bind to TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3, and regulate their activities by sequestering them in the ECM [Yamaguchi et al., 1990; Hildebrand et al., 1994; Takeuchi et al., 1994]. Thus, by analogy fibromodulin (or other SLRPs) and BMP-2 might regulate each other's actions at sites of bone formation during skeletal development. Since the biglycan knockout mice suggest that this postulate does not uniformly hold true, other



**Fig. 8.** In situ hybridization of calvariae. Fibromodulin and type I collagen antisense riboprobes were radiolabeled with  $^{35}$ S-UTP to a specific activity of at least  $10^8$  cpm/ $\mu$ g of template. Light field (A) and dark-field (B–C) photographs of calvariae at 15.5 dpc. B) fibromodulin and C) type I collagen expression.

Light field (D) and dark-field (E–F) photographs of calvariae at 18.5 dpc. E) fibromodulin and F) type I collagen expression. Hybridization was performed on sections from three embryos and representative sections are shown.

investigations will be necessary to dissect the mechanism of the potential functional redundancy of some of these molecules. Studies of the temporo-spatial expression of these molecules in the developing and maturing skeleton may help to elucidate why targeted ablation of some of these genes seems to result in no apparent skeletal phenotype. Based on similar temporo-spatial expression profiles of members of the small leucine-rich repeat proteoglycan family, the generation of selected double knockout mouse lines may ultimately be required to demonstrate the role and functional redundancy of these matrix proteins in the developing and maturing skeleton.

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