Modulation of bone morphogenetic protein signaling inhibits the onset and progression of ankylosing enthesitis

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Joint ankylosis is a major cause of disability in the human spondyloarthropathies. Here we report that this process partially recapitulates embryonic enchondral bone formation in a spontaneous model of arthritis in DBA/1 mice. Bone morphogenetic protein (BMP) signaling appears to be a key molecular pathway involved in this pathological cascade. Systemic gene transfer of noggin, a BMP antagonist, is effective both as a preventive and a therapeutic strategy in the mouse model, mechanistically interfering with enthesial progenitor cell proliferation in early stages of the disease process. Immunohistochemical staining for phosphorylated smad1/5 in enthesal biopsies of patients with spondyloarthropathy reveals active BMP signaling in similar target cells. Our data suggest that BMP signaling is an attractive therapeutic target for interfering with structural changes in spondyloarthropathy either as an alternative or complementary approach to current antiinflammatory treatments.

Introduction

The human spondyloarthropathies are a group of chronic inflammatory joint disorders with a prevalence of about 0.3% (1), which primarily affect individuals between 20 and 40 years of age. Different diagnostic entities, based on clinical, genetic, and pathological characteristics, are classified within this disease concept (2). These consist of ankylosing spondylitis, psoriatic arthritis, reactive arthritis, arthritis associated with inflammatory bowel disease, and a juvenile and an undifferentiated form of arthritis. Clinical manifestations include arthritis and enthesitis, which cause severe and permanent disability. Increasing evidence suggests that an anatomical zone referred to as the enthesis, where tendons and ligaments attach to underlying bone, is a primary target of the pathological process (3, 4). Enthesitis, synovitis, and subchondral bone changes are associated with enthesial cell proliferation and heterotopic cartilage and bone formation, all of which eventually lead to joint space bridging, a process called ankylosing enthesitis. Together, these features are considered the hallmark of spondyloarthropathy (3–5).

Long-term treatment strategies for chronic joint diseases aim to prevent tissue damage and loss of joint function. So-called disease modifying treatments not only target symptom control but are also intended to influence the pathological process itself. In spondyloarthropathy, conventional therapies, such as nonsteroidal antiinflammatory drugs and immune modulators, are considered symptom-controlling rather than disease-modifying treatments (6). TNF inhibitory agents have been introduced as biologic response modifiers (7, 8). Despite their clinical efficacy and widespread use, and in contrast to what is seen in rheumatoid arthritis, the disease-modifying properties of these strategies remain unclear. In chronic joint diseases such as rheumatoid arthritis and in animal models of these diseases (9), inflammation is associated with bone loss and destruction. In spondyloarthropathy, new bone formation is characteristic. Coupling of inflammation and subsequent ankylosis have not been demonstrated. Progressive ankylosis may persist despite absence of clinical disease activity (10). It is possible that continued suppression of inflammation may result in accelerated bone formation and ankylosis once the pathological cascade has been triggered. Therapeutic strategies specifically targeting cartilage and bone formation may therefore be required, either as an alternative or a complementary approach to immune-suppressing drugs, for gaining full control of the disease (11).

Bone morphogenetic proteins (BMPs), discovered to be proteins that ectopically induce a cascade of enchondral bone formation (12), play a crucial role in skeletal (13, 14) and joint morphogenesis (15–18). BMPs are members of the TGF-β superfamily, a group of polypeptides capable of regulating a wide array of cellular processes such as proliferation, differentiation, lineage determination, motility, and death (19, 20). In their canonical pathway, BMPs induce ligand-dependent type I and type II receptor heterodimerization, leading to phosphorylation of smad-signaling molecules (smad1/5) that bind smad4 and then translocate to the nucleus. BMP signaling is controlled at many levels, including that of extracellular antagonists such as noggin (19). Reactivation of embryonic-signaling pathways has been suggested as an essential part of repair and homeostasis in health and disease (21, 22). However, untimely or unwanted activation of signaling cascades fundamental for normal development may promote disease processes such as spondyloarthopathies. Therefore, we hypothesized that BMP signaling could play a direct role in joint ankylosis.

To address this, we studied BMP signaling in a spontaneous model of arthritis. Grouped caging of male DBA/1 mice led to the development of arthritis affecting hind paws and characterized by ankylosing enthesitis (23–25). This feature clearly distinguishes the model from other animal models of different types of joint disease such as spondyloarthropathies. Therefore, this method represents a useful system to study the role of BMP signaling in joint ankylosis.
diseases, such as rheumatoid arthritis and osteoarthritis, that are characterized by either synovial inflammation or progressive cartilage loss. Other features of the model include dactylitis and nail changes, symptoms characteristic of psoriatic arthritis (25). This model provides a tool for studying the mechanisms of joint ankylosis with potential relevance to the human spondyloarthropathies. In this report, we evaluate the effects of BMP signaling on initiation and progression of arthritis by gene transfer of noggin, a BMP antagonist.

Results

Distinct BMPs are expressed in ankylosing enthesitis. We used immunohistochemistry to identify specific BMPs, known to be involved in embryonic endochondral bone formation, in ankylosing enthesitis (Figure 1). The disease process was characterized by inflammation and proliferation at the enthesis, followed by cell differentiation into chondroblasts, prehypertrophic chondrocytes, and hypertrophic chondrocytes (24–27) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI23738DS1). The cartilage was progressively replaced by bone, which eventually led to joint ankylosis. Different BMPs were detected in distinct stages of ankylosing enthesitis as indicated by cell morphology (Figure 1). BMP2 was seen in spindle-shaped fibroblast-like cells in the proliferative zones as well as in more rounded chondroblasts in early disease stages (Figure 1, A and B). BMP2 was absent in prehypertrophic and hypertrophic chondrocytes (data not shown). In contrast, in later stages, BMP7 was largely restricted to prehypertrophic chondrocytes (Figure 1, D and E). BMP6 was found in hypertrophic chondrocytes (Figure 1, G and H).

Noggin gene transfer inhibits the onset and progression of spontaneous arthritis. We used intramuscular injections of plasmids that encode noggin cDNA under the control of a cytomegalovirus promoter to evaluate ubiquitous suppression of BMP signaling. Injection of 300 μg and 30 μg pcDNA3.1+noggin resulted in a dose-dependent increase in local mRNA expression (Figure 2A, A and B). In the RT-negative control samples, only very discrete amounts of residual noggin cDNA were detected after injection of 300 μg pcDNA3.1+noggin. Protein was detectable in muscle and serum (Figure 2C). Injection of 300-μg pcDNA3.1+ empty vector into the contralateral muscle did not result in locally or systemically detectable noggin. We chose to repeat injections every 3 weeks to ensure gene expression during the course of the experiment (28, 29). To ascertain that repeated injections of noggin cDNA did not result in the formation of antibodies that could block its effect, mice were injected 4 times with 300 μg of pcDNA3.1+noggin at 3-week intervals. After 15 weeks, no specific antibodies were found in their serum (data not shown).

We studied the effect of BMP inhibition on clinical incidence and severity of spontaneous arthritis. Male mice were caged together at the age of 12 weeks and monitored for clinical signs of arthritis until the age of 25 weeks. Animals were treated every 3 weeks (weeks 12, 15, 18, and 21) with plasmid injections (either 300 μg pcDNA3.1+noggin, 30 μg pcDNA3.1+noggin, or similar amounts of empty pcDNA3.1+). Injections of both 300 μg and 30 μg of noggin cDNA reduced the incidence of arthritis as compared with injections of empty vector controls (Gehan-Wilcoxon test, \( P < 0.05 \)) (Figure 2D). Similarly, pcDNA3.1+noggin reduced severity of arthritis as compared with empty vector (Mann-Whitney U test; \( P < 0.05 \)) (Figure 2E). The effect of noggin treatment on clinical severity during the course of the disease was evaluated by calculating the area under the curve of the clinical severity score (Figure 2F). Both noggin treatment groups (300 μg and 30 μg plasmids) showed a reduction in time-integrated clinical severity scores (Mann-Whitney U test, \( P < 0.05 \)). The effect was dose dependent. We noticed a slower disease onset and slightly reduced severity in the group that was treated with 300 μg empty vector as compared with the group treated with 30 μg empty vector. Yet this was not statistically significant. However, to exclude that the effect of pcDNA3.1+noggin treatment was due to nonspecific exogenous protein expression by gene transfer, additional mice were treated with pcDNA3.1+ coding for an irrelevant secreted protein (mouse Wnt5a with a mutated hydrophilic domain). No effect on arthritis was found (data not shown).

The effect of BMP inhibition as a therapeutic strategy was evaluated by pcDNA3.1+noggin or empty vector administration (300 μg) after symptoms of arthritis had developed. In a first set of experiments, individual mice were injected immediately after the
first symptoms (toe swelling or stiffness) were recognized. Mice were observed further for 3 weeks. Noggin plasmid treatment significantly reduced clinical disease progression, measured as the difference in clinical score between the beginning and end of the experiment, as compared with empty vector (Figure 3A) (Mann-Whitney U test, $P < 0.05$). In the noggin-treated group, 0 out of 6 mice showed disease progression (Figure 3B), and in 2 mice, improvement of arthritis was seen after treatment. In contrast, in the control group, disease progression was seen in 4 out of 8 mice (Figure 3B). In another set of experiments, individual mice were treated 10 days after the first onset of symptoms and further monitored for 3 weeks. Noggin gene therapy resulted in a significant reduction in clinical disease progression as compared with empty vector treatment (Figure 3C) (Mann-Whitney U test, $P < 0.05$). Of 9 mice, 4 in the noggin-treated group showed signs of clinical improvement, and in 1 animal, the clinical score increased (Figure 4D). In contrast, in the control group, 4 out of 8 mice showed an increase in clinical severity and none of the mice showed improvement of arthritis (Figure 4D).

**Noggin modulates endochondral bone formation in ankylosing enthesitis.** We studied the effect of exogenous noggin therapy on pathological bone formation by histological analysis in both the preventive and therapeutic experiments described above. Injection of pcDNA3.1+noggin (300 μg) every 3 weeks from the age of 12 weeks onward resulted in a significant difference in histological disease severity score as compared with injection of empty vector (Figure 4A) (Mann-Whitney U test, $P < 0.05$).

We analyzed the effect of noggin gene transfer in this experiment on different stages of endochondral bone formation (Table 1). In the pcDNA3.1+noggin–treated group, 5 out of 8 animals remained unaffected. Cell proliferation was seen in 3 out of 8 mice, but progression to cartilage formation was demonstrated in only 1 animal. Microscopic examination did not reveal bone formation or ankylosis. In contrast, 6 out of 8 animals in the control group were affected. Proliferation of enthesial cells was seen in all affected animals. Four animals showed signs of cartilage formation, and in 1 animal, we found bone formation leading to ankylosis.

We also evaluated histological disease severity when noggin cDNA was administered after the first appearance of symptoms. Noggin treatment (300 μg plasmid) resulted in a significantly lower disease severity score as compared with empty vector treatment (Mann-Whitney U test, $P < 0.05$) (Figure 4B). All animals from both groups showed positive histopathological findings (Table 1). In the pcDNA3.1+noggin-treated group, 5 out of 6 animals showed signs of cell proliferation, but only 2 out of 6 showed ectopic cartilage and bone formation. Ankylosis was documented in 1 animal. In contrast, 8 out of 8 animals from the control group showed cell proliferation, 6 out of 8 showed cartilage and bone formation, and 3 out of 8 showed joint ankylosis. Together, these observations suggest that suppression of BMP signaling by noggin can block early stages of pathological endochondral bone formation.

**Noggin gene transfer affects proliferation of progenitor cells.** We studied the target cell population for BMP signaling in ankylosing enthesitis (Figure 5). Immunofluorescent staining for nuclear phosphorylated
proinflammatory cytokines (TNF-α and IFN-γ) (Figure 6B), and BMP2, BMP6, and BMP7 (Figure 6C). Normal, affected, and arthritic joints 6 days after noggin gene therapy were tested. Significant differences in affected toes between normal and treated animals were present in mature or hypertrophic chondrocytes. No statistically meaningful differences were seen in BMP7 and IFN-γ expression.

**Active BMP signaling in human enthesial biopsies.** To specifically test whether our findings in the mouse are relevant to new cartilage and bone formation in human disease, we studied activation of BMP signaling in enthesial biopsies obtained from Achilles tendons of spondyloarthropathy patients (Figure 7A). Histological analysis revealed progenitor cell proliferation and cartilage formation strikingly similar to that in the mouse model (Figure 7, B and C). Activation of BMP signaling was apparent in the proliferating and differentiating cell population as revealed by the presence of phosphorylated-smad1/5 (Figure 7D). As in the mouse model, in later stages, this staining was negative (Figure 7D). Double staining for PCNA and phosphorylated smad1/5 reflected our observations in mice (Figure 7, E–G). Immunoreactivity for BMP2, BMP7, and BMP6 was recognized in proliferating spindle-shaped cells and in prehypertrophic and mature chondrocytes, respectively (Supplemental Figure 2).

**Discussion**

In this study, we demonstrate that BMP signaling affects both the initiation and the progression of ankylosis in a model of ankylosing spondylitis. Noggin gene transfer ameliorated the pathological cascade in ankylosing enthesitis. Noggin or empty vector control gene transfer was performed either as a preventive strategy (A–B) or as a therapeutic strategy immediately after the first symptoms appeared (C–D) after the first signs of arthritis had appeared. Disease progression, defined as the difference in clinical score between the beginning and the end of the experiment, was significantly reduced after noggin gene transfer as compared with that in empty vector controls. *Mann-Whitney U test; *P < 0.05, 3 weeks after injection. In B and D, individual clinical scores before and after treatment are shown.
losing enthesitis and spondyloarthropathy. BMP signaling has been studied in a mouse model of degenerative joint disease (30). Ectopic bone formation in this disorder may be a consequence of structural damage and is likely to be part of the body’s response to local injury, either as an attempt to increase joint stability or as a secondary effect of growth factors trying to preserve joint homeostasis (31). Our data provide evidence that deregulation of specific embryonic signaling pathways can also contribute to pathology. Indeed, several arguments indicate that the process of enthesal bone formation leading to ankylosis, as seen in spondyloarthopathies and in this model, is different from osteophytes in osteoarthritis. First, progressive ankylosis in both spine and peripheral joints is a major contributor to disability. Second, ankylosing enthesitis presents frequently as clinically symptomatic arthritis. Third, in contrast to osteoarthritis, heterotopic endochondral bone formation takes place in tissue that is considered one of the primary disease targets (3, 5, 32). Therefore, inhibition of ankylosis may be an important and specific therapeutic target in spondyloarthropathy (11, 33).

To address the mechanisms driving joint ankylosis, we have used a spontaneous mouse model of arthritis. Although it was originally reported as another model of rheumatoid arthritis (23), subsequent studies involving microscopic analysis have clearly demonstrated that this model is characterized by ankylosing enthesitis (25, 27) rather than destructive synovitis. This model is particularly attractive since arthritis occurs in association with environmental factors such as stress that have also been implicated in spondyloarthropathy (24, 34). It also shows specific features of psoriatic arthritis (25) that have not been reported in other models of arthritis. The high incidence and reproducibility of arthritis at the enthesis, characterized by the presence of neutrophils and some mononuclear cells, precedes or coincides with connective tissue proliferation and differentiation (25). Remarkably, Nordling et al. demonstrated that anti-idiotypic anticollagen type II antibody treatment may prevent the onset of arthritis (37). Autoimmunity toward components of fibrocartilage has been proposed by different authors as directly relevant for the development of spondyloarthropathy (5, 38). Autoantibodies toward type II collagen have also been demonstrated in spondyloarthropathy patients (39). Interestingly, we have also shown that inhibition of IFN-γ delays the onset of disease (26). However, in contrast to the effect of noggin, no therapeutic effect of anti-IFN-γ strategies was seen once symptoms had occurred (26). The finding that the disease process is

### Table 1

Effect of noggin gene transfer on different features of ankylosing enthesitis

<table>
<thead>
<tr>
<th>Prevention</th>
<th>Normal Proliferation</th>
<th>Cartilage formation</th>
<th>Bone formation</th>
<th>Joint ankylosis</th>
</tr>
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<td>Empty vector</td>
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<td>6/8 (75)</td>
<td>4/8 (50)</td>
<td>1/8 (12)</td>
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<td>3/8 (37)</td>
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<td>8/8 (100)</td>
<td>6/8 (75)</td>
<td>6/8 (75)</td>
</tr>
<tr>
<td>Noggin</td>
<td>0/6 (17)</td>
<td>5/6 (83)</td>
<td>2/6 (33)</td>
<td>2/6 (33)</td>
</tr>
</tbody>
</table>

Data are shown as the number of animals exhibiting a specific trait as compared with the total number of animals in the group. Percentages are shown in parentheses.

in this model, in contrast to other models of ankylosis (35), highlight its usefulness for mechanistic studies in the process of ankylosis in spondyloarthropathy. As no animal model completely mimics human disease, different questions can be raised regarding the relevance of this model to human spondyloarthropathy. First, the absence of spine pathology in this model can at least partially be explained by differences in weight bearing between rodents and humans. So far, there are no indications that ectopic bone formation in spine and in peripheral joints is distinct. Both processes mimic predominantly endochondral bone formation (5, 36). Second, the inflammatory reaction associated with the proliferative and meta-plastic process at the enthesis is still poorly defined. We have demonstrated that dactylitis or local inflammation

Figure 5

Noggin inhibited BMP signaling in spontaneous arthritis. (A) Immunofluorescent staining for phosphorylated smad1/5/8 demonstrating positive nuclei (red staining, arrowheads; detail in inset) in the zone of proliferating cells. The area corresponding to hypertrophic chondrocytes is negative (arrows). (B) Phosphorylation of smad1/5/8 was increased in arthritic as compared with healthy interphalangeal joints and was inhibited by noggin gene transfer as shown by semiquantitative Western blot (n = 5 animals per group, Kruskal-Wallis test P < 0.05; Mann-Whitney U test: *P < 0.05 versus normal joints; **P < 0.05 versus untreated affected joints). (C) Double immunofluorescence staining for phosphorylated smad1/5 (red) and PCNA (green) demonstrating nuclear colocalization (yellow in overlay). Scale bars: 50 μm.
apparently T cell independent seems to be in contrast with the proposed role for T cells in both psoriatic arthritis (40) and ankylosing spondylitis (5). In these experiments, however, T cell receptors αβ−/− and γδ−/− were used separately. Therefore, a role for T cells in the development of autoimmunity toward fibrocartilage components in this model cannot be excluded. Taken together, all these data support the concept of juxtaposed stages in the disease that are coupled but partially independent. Our model therefore seems representative of the process of ankylosis in spondyloarthropathy, which can be considered as a specific therapeutic target.

The somewhat surprising effects in the early stages of the disease highlight the importance of BMP signaling. This pathway is an essential part of the complex network regulating skeletal development (14–18). Endochondral bone formation is initiated by mesenchymal cell condensations. Cells within these condensations undergo chondrogenic differentiation, progressively acquiring the phenotypes of proliferating, prehypertrophic, and hypertrophic chondrocytes. In the later stages, the cartilaginous elements are replaced by bone (13). Studies in developmental models suggest that fine-tuned balances between BMPs and inhibitors such as noggin appear to influence different stages of endochondral bone formation. Retroviral over- and misexpression of noggin in the developing chick limb revealed a dual role for BMP signaling in early stages (41). Both cell condensation and differentiation of chondroprogenitor cells into chondrocytes were inhibited effectively, depending on the time of infection.

Various challenges regarding the role of BMPs in ankylosing enthesitis remain. First, understanding of the relative contribution of specific BMPs and inhibitors to the pathological cascade needs to be refined. Our data suggest an early role for BMP2 and later involvement of BMP6 and BMP7. The presence of additional ligands has not been studied. However, regardless of their presence, noggin has been demonstrated as an antagonist of the BMP2/4, the BMP5/6/7, and the growth and differentiation factor 5/6/7 groups (42). Therefore, noggin overexpression is likely to change the overall balance in BMP signaling in our model. Second, the identification of factors leading to the activation of the BMP-signaling pathway is of critical importance to understanding the links between inflammation and bone formation, but the exact mechanism remains unknown. We and other groups have demonstrated that specific BMPs, including BMP2 and BMP6, are upregulated by proinflammatory cytokines in cell populations obtained from the joint, providing circumstantial evidence that inflammatory changes and BMP expression are linked (43–45). Other explanations include increased expression of BMP receptors or BMP-signaling enhancers (46), through genetic or environmental factors, that lead to enhanced activation of the smad pathway.

It is well known that the biological response to BMPs is dependent on the target tissue (19). Injection of recombinant BMP2 into the knee joints of mice resulted in osteophyte formation in areas adjacent to the bone and did not result in cartilage formation in the synovium itself (47). Adenoviral infection of periost with BMP2 in vivo stimulated endochondral bone formation at the application site (48).

In summary, our findings support the concept that BMP signaling is an attractive therapeutic target for achieving disease modification in spondyloarthropathy. Symptom control by inhibition of inflammation may not be sufficient to stop the structural progression of disease and the resulting disability. Therefore, specific molecular targets involved in cartilage and bone forma-
tion, including the BMPs and their transduction machinery, may provide a complementary or alternative therapeutic approach in patients with spondyloarthropathies. In the complex network of cell differentiation and lineage commitment, molecular networks may be crucial, and single-gene approaches to treating diseases may not be sufficient.

**Methods**

*Animal experiments.* Three sets of experiments were performed. First, male DBA/1 mice (Jackson Laboratory or Janvier) from different litters were mixed and caged together in groups of 4 to 6 mice at the age of 12 weeks. Full-length mouse nogen cDNA (a gift of R. Harland, University of California, Berkeley, California, USA) was cloned into pcDNA3.1+ vector (Invitrogen Corp.). Either 300 or 30 μg of pcDNA3.1+nogen or pcDNA3.1+ was injected into the tibialis anterior muscles at weeks 12, 15, 18, and 21. Animals were sacrificed at 25 weeks. Second, after the first symptoms appeared in each mouse, pcDNA3.1+nogen (300 μg/ml) or empty vector was injected immediately. Third, animals with clinical signs of arthritis were treated with pcDNA3.1+nogen (300 μg/ml) or empty vector 10 days after the first symptoms were noted. In these experiments, individual mice were killed 3 weeks after injection of plasmid cDNA. The Ethics Committee for Animal Research (Katholieke Universiteit Leuven) approved all animal experiments. All experiments and analyses were run in a randomized and blinded fashion.

**Human materials.** Enthesial biopsies were obtained during surgery for refractory enthesitis. The Ethics Committee for Human Medical Research (Katholieke Universiteit Leuven) approved all procedures, and patient informed consent was obtained.

**Immunohistochemistry and immunofluorescence.** For immunohistochemistry and immunofluorescence, sections were quenched with 3% H2O2/H2O or 50 mM NH4Cl respectively and preincubated with donkey serum (20% in Tris-buffered saline [TBS]). Sections were incubated with polyclonal primary antibodies against BMP2 (Pfizer) (5 μg/ml), BMP7 (Pfizer) (10 μg/ml), BMP6 (Santa Cruz Biotechnology Inc.) (10 μg/ml), phosphorylated smad1/5/8 (PS1) (P. Ten Dijke, University of Leiden, Leiden, The Netherlands) (1/100) overnight at 4°C. Anti-BMP2 antibody was raised against peptide sequence Ac-REKRQAKHKKRRKKSSC-NH2. BLAST protein analysis (http://www.ncbi.nlm.nih.gov/BLAST) showed that this peptide is specific for BMP2 and is conserved across species (human, mouse, rat, xenopus). Anti-BMP7 antibody was raised against peptide sequence Ac-TGSKQRSQTRSkTPKCN-H2. This peptide sequence is specific for human BMP7, as shown by BLAST protein analysis. The mouse and rat BMP7 sequence showed 1 amino acid difference (G instead of S in position 3). Anti-BMP6 was raised against a peptide sequence from the ammino-terminus of human BMP6. Antiphosphorylated smad1/5 antibody recognizing the phosphorylated C-tail in Smad1 was generated by injection of peptide KKK-NPISVS containing 2 C terminal phosphoserine residues coupled to keyhole limpet hemocyanin (49). Negative controls were performed with species-specific IgG (Jackson ImmunoResearch Laboratories Inc.) or preincubation of the antibodies with blocking peptides or proteins. After washing and a second blocking step, sections were incubated with horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) (1/100). For immunofluorescence, secondary antibodies were Cy3-conjugated goat anti-rabbit antibody (1/100 dilution) or Cy2-conjugated anti-goat antibody (1/100 dilution) (Jackson ImmunoResearch Laboratories Inc.).

**Western blot analysis.** For phosphorylated smad1/5, interphalangeal joints were dissected, frozen, thawed twice, and stored in 6 M urea/10 mM Na2CO3 for 30 min and then dissolved in Laemmli buffer (25). Equal amounts of total protein were separated by 8% SDS-PAGE and blotted onto nitrocellulose membranes. Equal protein loading was confirmed by Coomassie blue staining. Blots were blocked for 1 h with 5% nonfat dry milk in TBS-T. After blocking, membranes were incubated overnight with 1:1000 dilutions of rabbit polyclonal antibodies against phosphorylated smad1/5 (PS1) (P. Ten Dijke, University of Leiden, Leiden, The Netherlands) or mouse monoclonal antibodies against rat and human Smad4. Blots were then incubated with horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) (1/100). Blots were developed with chemiluminescence according to the manufacturer’s instructions (Amersham Bioscience) and exposed to X-ray film (Kodak). All protein bands were determined using a digital imaging system and analyzed using ImageJ software (University of Wisconsin, Madison, WI). Data are given as mean ± SEM and were compared statistically using the Student’s t test. A p value of less than 0.05 was considered significant.

**Histology and immunohistochemistry.** Hematoxylin and eosin–stained sections were used for histological analysis. Implant masses were examined for bone formation, cartilage formation, and ankylosis. Sections were immunostained for calcification using Alizarin Red S and for proliferation using 5-bromo-2′-deoxyuridine (BrdU).For histological examination, hind paw forefeet were formalin-fixed, decalcified using Decal (Serva), paraffin embedded, and cut in a transverse plane. A histological score was developed previously (25, 26): 0, no symptoms; 1, redness and swelling in one toe; 2, redness and swelling in more than one toe; 3, toe stiffness; and 4, deformity or ankle involvement. Both hind paws were evaluated, resulting in a maximum score of 8. For histological examination, hind paw forefeet were formalin-fixed, decalcified using Decal (Serva), paraffin embedded, and cut in a transverse plane. A histological score was developed previously (25, 26): 0, no symptoms; 1, inflammatory cell infiltration; 2, enthesal fibroblast-like cell proliferation; 3, cartilage formation; 4, bone formation; and 5, ankylosis. In each mouse, a cumulative score for all interphalangeal and metatarsophalan-geal joints of the 2 hind limbs was calculated by summation of the individual joint scores. Both clinical and pathological scores were found to be consistent and reproducible with little intra- and interobserver variability.

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Tris/ph 7.8. Five-hundred ng protein, diluted in 4× NuPAGE-LDS buffer (Invitrogen Corp.) was analyzed under reduced conditions (2% mercaptoethanol and 0.1 M DTT) in NuPAGE-MES-SDS buffer (Invitrogen Corp.). Proteins were transferred onto a PVDF membrane in 0.4 M glycine/0.5 M Tris-base/0.01 M SDS and 200 ml/l methanol. Blots were probed with antibody PS1 (1/2500 in TBS-Triton/3% milk) and washed with TBS-Triton, and goat anti-rabbit horseradish peroxide-conjugated secondary antibody (1/5000 in TBS-Triton/3% milk) (Jackson Immunoresearch Laboratories Inc.) was added. For visualization, we used SuperSignal Pico Chemiluminescent Substrate (Pierce).

For noggin, tibialis anterior muscles were injected with 300 μg pcDNA3.1+noggin or empty vector. After 72 hours, muscles and blood were collected. Muscles were extracted in 1 M guanidium hydrochloride/50 mM Na-acetate overnight. Samples were desalted, lyophilized, and redissolved in 200-μl immunoprecipitation buffer (150 mM NaCl/1% NP-40/0.2 mM EDTA/50 mM NaF/1 mM Na3PO4/20 mM Tris/ph 7.6). Samples and serum were incubated overnight at 4°C with 2 μg goat anti-noggin antibody. Protein A/G sepharose solution (Amersham Biosciences) was used for precipitation. Pellets were dissolved in loading buffer-8 M urea. Western blot was performed as above with goat anti-noggin polyclonal affinity purified antibody (R&D Systems) (1/5000) and horseradish peroxidase-conjugated mouse anti-goat (1/20000) (Jackson Immunoresearch Laboratories Inc.). Recombinant noggin-Fc (Invitrogen Corp.) was used as a positive control.

PCR RNA was isolated from tibialis anterior muscle or interphalangeal joints using Trizol (Invitrogen Corp.) and reverse transcribed (Superscript III; Invitrogen Corp.). For conventional PCR, the following primers were used: β-actin forward primer 5′-TGAGGGGTCACCCCCAACGGCACCATTCT-3′; β-actin reverse primer 5′-CTAGAAGACCTTGGTGGAGATG-3′; noggin forward primer 5′-GACATGAGGCGTCGCCCACG-3′; noggin reverse primer 5′-GAGGACGCGAGGCACAC-3′. For β-actin, a 3-step PCR was performed with an annealing temperature of 60°C; for noggin, a 2-step PCR was performed with an annealing temperature of 72°C. For real-time PCR, gene expression was studied using Assay-on-Demand primer-probe systems (Applied Biosystems). Expression levels were normalized to GAPDH expression using the comparative threshold cycle method.

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