Cooperative activity of noggin and gremlin 1 in axial skeleton development

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SUMMARY
Inductive signals from adjacent tissues initiate differentiation within the somite. In this study, we used mouse embryos mutant for the BMP antagonists noggin (Nog) and gremlin 1 (Grem1) to characterize the effects of BMP signaling on the specification of the sclerotome. We confirmed reduction of Pax1 and Pax9 expression in Nog mutants, but found that Nog;Grem1 double mutants completely fail to initiate sclerotome development. Furthermore, Nog mutants that also lack one allele of Grem1 exhibit a dramatic reduction in axial skeleton relative to animals mutant for Nog alone. By contrast, Pax3, Myf5 and Lbx1 expression indicates that dermomyotome induction occurs in Nog;Grem1 double mutants. Neither conditional Bmpr1a mutation nor treatment with the BMP type I receptor inhibitor dorsomorphin expands sclerotome marker expression, suggesting that BMP antagonists do not have an instructive function in sclerotome specification. Instead, we hypothesize that Nog- and Grem1-mediated inhibition of BMP is permissive for hedgehog (Hh) signal-mediated sclerotome specification. In support of this model, we found that culturing Nog;Grem1 double-mutant embryos with dorsomorphin restores sclerotome, whereas Pax1 expression in smoothed (Smo) mutants is not rescued, suggesting that inhibition of BMP is insufficient to induce sclerotome in the absence of Hh signaling. Confirming the dominant inhibitory effect of BMP signaling, Pax1 expression cannot be rescued in Nog;Grem1 double mutants by forced activation of Smo. We conclude that Nog and Grem1 cooperate to maintain a BMP signaling-free zone that is a crucial prerequisite for Hh-mediated sclerotome induction.

KEY WORDS: Sclerotome, BMP antagonist, Hedgehog signaling, Mouse

INTRODUCTION
Somics are bilateral metameric structures that form from the mesoderm that flanks the neural tube and notochord. Starting as uncommitted epithelial spheres, somites rapidly differentiate into the dorsal-lateral dermomyotome, which contains the precursors for skeletal muscles and dermis, and ventral-medial sclerotome, which gives rise to the bone and cartilage of the axial skeleton. Embryological and genetic analyses have identified the inductive interactions and molecular signals that initiate somite pattern formation. Sclerotome is specified by sonic hedgehog (Shh) secreted from the notochord and floor plate. These tissues, or Shh alone, induce sclerotome marker expression in cultured paraxial mesoderm (Borycki et al., 1998; Fan and Tessier-Lavigne, 1994; Marcelle et al., 1999; Murtaugh et al., 1999; Teillet et al., 1998). Furthermore, Shh mutant embryos fail to form an axial skeleton (Chiang et al., 1996) and sclerotome markers are not expressed in embryos mutant for the obligate hedgehog (Hh) signal-activating receptor smoothed (Smo) (Zhang et al., 2001). Embryos lacking both Gli2 and Gli3, which along with Gli1 are the major transcription factors that mediate Hh signaling, also fail to form sclerotome (Buttitta et al., 2003). Shh induces expression of the sclerotome-specific transcription factors Pax1 and Pax9, which have redundant roles in the formation of the vertebral column (Peters et al., 1999).

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The effect of Hh signaling on sclerotome development depends stage specifically on BMP signaling. Chick presomitic mesoderm (PSM) explants initiate cartilage marker expression in response to BMP through a Sox9 and Nkx3.2 autoregulatory loop (Zeng et al., 2002), provided that the tissue has first been exposed to Shh (Murtaugh et al., 1999). In addition, BMP-secreting cells induce the formation of superficial dorsal cartilage (Monsoro-Burq et al., 1996). By contrast, early BMP exposure interferes with vertebral development. Treatment of chick PSM with BMP4 blocks all sclerotome and chondrocyte marker expression (Murtaugh et al., 1999), whereas ventrally positioned BMP4-expressing cells prevent formation of lateral aspects of the vertebra (Monsoro-Burq et al., 1996). Mice lacking the secreted BMP antagonist noggin (Nog) provide genetic evidence for the variable effect of BMP on the development of the axial skeleton; Nog mutant embryos exhibit delayed sclerotome marker expression and loss of caudal vertebrae but an excess of cartilage formation in anterior ribs and vertebrae (Brunet et al., 1998; McMahon et al., 1998).

Although the Nog phenotype demonstrates the importance of BMP antagonist function in axial skeletal development, our understanding is incomplete. Caudal vertebral development in Nog mutants is partially restored by a reduction in BMP signaling (Wijgerde et al., 2005), showing that caudal sclerotome development depends on the local integration of BMPs and Nog. High expression of Bmp4 in the posterior lateral plate might dictate a need for Nog in the nearby somites, but there might be no requirement for such regulation in the anterior domain. Additionally, the mechanism by which Nog promotes sclerotome specification is not defined. Based on the capacity of Nog to induce Pax1 expression in the presence of reagents that inhibit Hh signal transduction, it has been suggested that Nog might induce sclerotome independently of Hh (McMahon et al., 1998).
Furthermore, although it is clear that BMP can inhibit sclerotome specification, the mechanism underlying this suppression is unknown.

Multiple BMP antagonists are expressed during mouse gestation, and compensation by other antagonists might explain the variable consequences of Nog mutation for axial skeleton development. Nog;Chrd double mutants exhibit deficiencies in the derivatives of anterior mesoderm, including cervical vertebrae (Bachiller et al., 2000). However, this was attributed to a lack of rostral Shh and is therefore distinct from the defect exhibited by Nog mutants, which have a notochord and express Shh (McMahon et al., 1998). Thus, the question of whether multiple antagonists contribute to sclerotome development remains open.

The DAN family member antagonist gremlin 1 (Grem1) is expressed in PSM and newly formed somites starting at E8.5 (Pearce et al., 1999) and is a candidate factor for regulating axial skeletal development. Although Grem1 mutants have no apparent somite phenotype (Khokha et al., 2003), we hypothesized that ablating Grem1 in combination with Nog might result in more severe consequences for somite pattern formation. We show that removing both Nog and Grem1 causes the loss of the sclerotome. We also demonstrate that the elevated BMP signaling in these mutants affects the outcome of the Hh signals that underlie sclerotome development.

MATERIALS AND METHODS

Generation of mice

A conditional Nog targeting construct was made using genomic DNA clones previously used to make the Nog null mutant (McMahon et al., 1998). Briefly, a pgk-Neo cassette flanked by FRT sites and followed by a single lox site was inserted into the unique EcoRI site 1959 bp downstream of the ATG initiation codon. An XbaI site followed by a lox site was inserted into the unique NotI site located 252 bp upstream of the ATG initiation codon. A pgk-diphtheria toxin cassette was located upstream of Neo in embryonic stem (ES) cells and colonies were selected for resistance to G418. Positive colonies were subjected to Southern analysis with a 400 bp product is generated using 5'-GGGAAGGGG-3' and 3'-AGGCTCCGCACAGAGAAACAAG-3'.

RESULTS

Expression of Nog and Grem1 during somite patterning

In the developing mouse somite, the expression of markers of sclerotome and dermomyotome initiates from early embryonic day (E) 8.5 to E9.0. At these stages, Nog and Grem1 are expressed in the midline and somites, respectively, consistent with roles in somite patterning (McMahon et al., 1998; Pearce et al., 1999). We revisited the expression of Nog and Grem1 between E8.5 and E9.0 with an emphasis on the developing trunk (Fig. 1). E8.5 embryos expressed Nog throughout the notochord and dorsal neural tube (Fig. 1A, A'). Immediately following turning, Nog expression was diminished in the anterior notochord but remained in the posterior notochord and the roof plate (Fig. 1B). This pattern persisted to E9.5, at which point Nog expression initiated in the medial lip of the dermomyotome (Fig. 1C). Grem1 was expressed at E8.5 in recently formed somites as well as in presomitic and lateral plate mesoderm and posterior dorsal neural tube (Fig. 1D, D'). As the embryo initiated turning Grem1 expression became restricted to the lateral dermomyotome (Fig. 1E). Similar to Nog, Grem1 expression could also be detected in the E9.5 medial dermomyotome (Fig. 1F).

This analysis confirms that, although not expressed in overlapping domains, Nog and Grem1 could potentially have overlapping functions in early somite patterning.

Generation of Nog and Grem1 mouse mutants

Although capable of forming somites and initiating early somite patterning, Nog mutants exhibit clear deficiencies in the development of somite-derived tissues (McMahon et al., 1998). Based on their concurrent expression and similar molecular properties (Hsu et al., 1998; Zimmerman et al., 1996), we hypothesized that mutations in Grem1 might enhance the Nog mutant phenotype. To increase the recovery of mutants, we used conditional mutants to test the additive effect of Grem1 ablation on the somite phenotype in Nog mutants.

Using homologous recombination in ES cells, we introduced loxP elements to generate a conditional allele of Nog that would be deleted in cells containing the second lox site located 1959 bp downstream of the ATG initiation codon. A pgk-diphtheria toxin cassette was located upstream of Neo in embryonic stem (ES) cells and colonies were selected for resistance to G418. Positive colonies were subjected to Southern analysis with a 400 bp product is generated using 5'-GGGAAGGGG-3' and 3'-AGGCTCCGCACAGAGAAACAAG-3'.

Quantitative PCR

cDNA was synthesized from Trizol-extracted total RNA using standard methods. Expression was assessed by quantitative (Q) PCR with SYBR Green reagents on an Applied Biosystems 7300 system using β-actin as an internal reference. Primer sequences are provided in Table S1 in the supplementary material.
mutants had the characteristic deformed limbs (Khokha et al., 2003). At E16.5, phenocopied conventional nulls (see Fig. S1 in the supplementary material). Female homozygotes (McMahon et al., 1998).

heterozygous males (Gazzerro et al., 2007). Female homozygotes (fx/fx embryos were crossed to heterozygous males (Nog+/H11032;Grem1fx/+) that were also homozygous for constitutively active β-actin-Cre (Lewandoski et al., 1997). Thus, all offspring receive a single β-actin-Cre transgene and half the embryos are homozygous for the conditional allele. The homozygous conditional Nog and Grem1 mutants phenocopied conventional nulls (see Fig. S1 in the supplementary material). At E16.5, Nog homozygous conditional mutants exhibited the characteristic broad autopods, neural tube defects and shortened tails (McMahon et al., 1998). Grem1 homozygous conditional mutants had the characteristic deformed limbs (Khokha et al., 2003). QPCR confirmed efficient loss of each allele. In Grem1fx/embryos, Grem1 expression was 0.015% that of Grem1fx/+ littermates. Nog expression in Nogfx/embryos was less than 0.010% of Nogfx/+ levels.

To generate embryos lacking both Nog and Grem1, female double homozygotes (Nogfx/fx;Grem1fx/+) were crossed to double-heterozygous males (Nogfxfx;Grem1fx/) that were homozygous for β-actin-Cre. This strategy increases the number of double mutants 4-fold over a strategy involving intercrossed double-heterozygous null mutants, producing equal numbers of the following four genotypes: Nogfxfx;Grem1fx/, used as controls; Nogfxfx;Grem1fx/+, also referred to as Nog mutants; Nogfxfx;Grem1fx/+, also referred to as Grem1 mutants; and Nogfxfx;Grem1fx/+, also referred to as double mutants.

Nog and Nog;Grem1 mutant embryos have impaired somite formation

For dissections prior to E9.5, we collected embryos of the four possible genotypes at approximately the expected 1:1:1:1 ratio (see Fig. S1 in the supplementary material). We recovered fewer double mutants from later dissections; at E9.5, we obtained 65% of the predicted frequency of double mutants, whereas the other classes were represented as expected (see Fig. S1D in the supplementary material). Nogfx/fx;Grem1fx/+ embryos appeared normal at all stages examined and throughout adulthood (Fig. 2A). There are no reported defects in embryos lacking Grem1 prior to E10.5. Likewise, Nogfx/+;Grem1fx/fx embryos appeared as wild type at E8.5-9.5 (Fig. 2B). On this background, the phenotype of Nogfx/fx;Grem1fx/+ embryos resembled that of homozygous Nog null mutants (McMahon et al., 1998), exhibiting small posterior somites and buckled neural tubes that frequently failed to close anteriorly (Fig. 2C). The morphological defects of double-mutant embryos (Nogfx/fx;Grem1fx/+) were invariably more severe than those observed in Nogfx/fx;Grem1fx/+ siblings (Fig. 2D). The neural tube was highly kinked and both the neural tube and brain failed to fuse dorsally. In contrast to Nogfx/fx;Grem1fx/+, central nervous system development anterior to the midbrain arrested in the double mutants. Owing to arrested growth caudal to the thoracic trunk, double-mutant embryos were shorter than the other genetic classes.

To characterize the consequences of Nog;Grem1 mutation for somite formation, we assessed the expression of Meox1, which encodes a transcription factor that is initially expressed throughout the epithelial somite (Candia et al., 1992) and, along with Meox2, is required for the chondrogenesis and myogenesis of paraxial mesoderm (Mankoo et al., 2003). At E9.5, double-heterozygous control and Grem1 mutant (Nogfx/+;Grem1fx/+) embryos expressed Meox1 throughout the somites (Fig. 2E,F). In Nog mutants, somitic Meox1 expression at E9.5 highlighted the characteristic progressive reduction in somite size in the lumbar region, although expression was present in the hindlimb region (Fig. 2G). In double mutants, the caudal expression domain was also lost (Fig. 2H). Consistent with a requirement for Grem1 in this region, Grem1 was expressed normally in the PSM and was still present in Nog mutant embryos (not shown).

Some of the differences in somite size were already apparent at E9.0, although the severity of reduction in the Nog homozygotes at somites 8-12 was not increased by ablation of Grem1 (Fig. 2J,K). Transverse sections showed disordered Meox1 staining extending over a smaller area in both Nogfx/fx;Grem1fx/ and Nogfx/fx;Grem1fx/ embryos relative to controls. To better characterize somite morphology, we stained somites for filamentous actin (F-actin), which marks the internal surface of the epithelial somite. The somitocoele was clearly demarcated in control sections (Fig. 2L). Nog and double-mutant somites showed disorganized F-actin accumulation within the somite, suggesting that epithelium formation is compromised in the thoracic somites of these embryos (Fig. 2M,N). Equivalent results were obtained with samples stained with an antibody to atypical protein kinase C (Fig. 2L-N). We conclude that Nog is required for the formation of the first 15-20 somites and that Grem1 mutation does not exacerbate this somitogenesis phenotype. By contrast, in the absence of Nog, Grem1 is sufficient to allow axis elongation and paraxial mesoderm development in posterior aspects of the embryo.
expression of sclerotome markers. Paired domain transcription factor family member Pax1 is one of the earliest markers of sclerotome and is required for normal vertebral development (Wilm et al., 1998). As expected, Nog\textsuperscript{fx/fx}\texttimes\texttimes\texttimes Pax1\textsuperscript{fx/fx} control and Grem1 mutant (Nog\textsuperscript{fx/fx}\texttimes\texttimes\texttimes Pax1\textsuperscript{fx/fx}) embryos exhibited normal Pax1 expression from E8.5 to E9.5 (Fig. 3A,B). Pax1 transcript was also detected in E9.5 Nog mutant (Nog\textsuperscript{fx/fx}\texttimes\texttimes\texttimes Grem1\textsuperscript{fx/fx}) somites, although, as with Mox1, expression was not present posterior to somite 15 (Fig. 3C). However, embryos mutant for both Nog and Grem1 exhibited a striking loss of somitic Pax1 expression at all stages examined, from E8.5 to E9.5 (Fig. 3D). Most (11 of 18) had no detectable somitic Pax1 expression, whereas others showed residual, small expression domains within the five anteriormost somites. Pax1 expression in the pharyngeal arches was present in all genetic classes.

We also analyzed the expression of Pax9, Nkx3.2, Uncx4.1 (Uncx – Mouse Genome Informatics) and Tbx18 in Nog\textsuperscript{fx/fx}\texttimes Pax1\textsuperscript{fx/fx} double-mutant embryos. Compared with Pax1, we observed an even greater effect of Nog and Grem1 mutation on Pax9 activation, which normally exhibits similar expression to Pax1. In contrast to the control and to Nog mutant or Grem1 mutant embryos (see Fig. S2A-C in the supplementary material), we never observed Pax9 expression in the somites of double mutants (see Fig. S2D in the supplementary material; n=5). However, expression remained in the pharyngeal arches and tail of all classes. Somitic expression of Nkx3.2, which in later axial skeleton development requires BMP for induction of cartilage genes (Zeng et al., 2002), was also absent specifically in Nog\textsuperscript{fx/fx}\texttimes\texttimes\texttimes Grem1\textsuperscript{fx/fx} embryos (see Fig. S2E-H in the supplementary material; n=4). Uncx4.1 is required for the formation of pedicles, transverse processes and proximal ribs, which are derivatives of the lateral sclerotome (Leitges et al., 2008).
2000). In the first 12-15 somites at E9.5, we observed Uncx4.1 expression in the posterior compartment of the sclerotome of Nog^{fx/};Grem1^{fx/+}, Nog^{fx/+};Grem1^{fx/} and Nog^{fx/};Grem1^{fx/+} embryos. No expression was detected in the lumbar trunk of Nog^{fx/};Grem1^{fx/} embryos, but expression was detected at the level of the hindlimbs. By contrast, expression in Nog^{fx/+};Grem1^{fx/+} was either absent (2 of 5) or sharply reduced (see Fig. S2L in the supplementary material). The trunk, Tbx18 marks the anterior epithelial somite (Kraus et al., 2001) and Tbx18 loss of function results in reduced pedicles and proximal ribs (Bussen et al., 2004). We observed somitic Tbx18 expression in Nog^{fx/};Grem1^{fx/+}, Nog^{fx/+};Grem1^{fx/} and Nog^{fx/};Grem1^{fx/+} embryos (see Fig. S2M-O in the supplementary material). In Nog^{fx/+};Grem1^{fx/} embryos (see Fig. S2P in the supplementary material) somitic expression was markedly reduced (n=3) or absent (n=1). As both Tbx18 and Uncx4.1 are expressed throughout the respective anterior and posterior somite halves before becoming confined to the sclerotome, expression of these genes in some double mutants is not inconsistent with sclerotome deficiency. Rather, this analysis suggests that somite anterior-posterior pattern formation occurs in Nog^{fx/+};Grem1^{fx/} embryos.

We next sought to rule out the possibility that Nog^{fx/+};Grem1^{fx/} mutants have a generalized defect in somite differentiation. Another paired domain family member, Pax3, is expressed in the dorsal neural tube and dermomyotome at E9.5. In contrast to sclerotome markers, Pax3 was expressed in the somites of all genetic classes (Fig. 3E-H). Control and Grem1 mutant embryos showed normal E9.5 Pax3 expression. Both Nog mutant (Nog^{fx/};Grem1^{fx/+}) and Nog^{fx/+};Grem1^{fx/} double-mutant (n=8) embryos showed diminished and disordered Pax3 expression in the dermomyotome, reflective of compromised somite formation. Nevertheless, all embryos in both classes showed Pax3 expression. Furthermore, the determinant of proliferating myoblasts, Myf5, was also expressed in Nog;Grem1 double-mutant embryos (n=5), indicating that the early skeletal muscle program is maintained in the absence of these antagonists (see Fig. S3 in the supplementary material). To test the capacity of the double-mutant embryos to form lateral dermomyotome, we examined expression Lbx1, which encodes a transcription factor that marks myoblasts fated to form the skeletal muscle of the limbs. Similar to the other dermomyotomal markers analyzed, Lbx1 was expressed at E9.5 in all genetic classes (see Fig. S3 in the supplementary material; n=5).

Although the expression of these transcripts was altered relative to controls, their presence indicates that Nog^{fx/+};Grem1^{fx/} double mutants can form dermomyotome. Based on our analysis of tissue-specific markers, we conclude that deletion of Nog and Grem1 specifically prevents induction of the sclerotome lineage in the recently formed somite. This phenotype is distinct from the somitogenesis defect shared by Nog and Nog;Grem1 double mutants and is a novel instance of BMP antagonists with non-overlapping expression domains serving a cooperative function in organogenesis.

Later effects of antagonist mutation on sclerotome derivatives

The expected outcome of the failure to form sclerotome is the absence of an axial skeleton. However, Nog;Grem1 double mutants do not survive to chondrogenic stages. We therefore tested the chondrogenic potential of Nog^{fx/+};Grem1^{fx/+} somites in micromass culture. Whereas Nog^{fx/+};Grem1^{fx/+} cultures showed numerous chondrogenic nodules after 6 days, Nog^{fx/+};Grem1^{fx/} tissue failed to form any cartilage (see Fig. S4 in the supplementary material). We reasoned that differences in axial skeletal development among the genotypes that do survive to fetal stages might substantiate these findings in vivo. To define the contribution of Grem1 heterozygosity to the Nog mutant phenotype, we stained E13.5 embryos with Alcian Blue to reveal cartilage morphology (Fig. 4). Nog^{fx/+} specimens were compared with the skeletons of Nog^{fx/+};Grem1^{fx/} animals, as well as with those of Nog^{fx/+};Grem1^{fx/+} controls and Nog^{fx/+};Grem1^{fx/+} mutants. As the severity of the Nog mutant phenotype varies between strains, we were careful to maintain equivalent genetic backgrounds (Tylzanowski et al., 2006) (see Materials and methods).

Relative to Nog^{fx/+};Grem1^{fx/+} and Nog^{fx/+};Grem1^{fx/+} specimens, which exhibited apparently normal development of the axial skeleton (Fig. 4A,B), Nog^{fx/+} mutants exhibited reduced ribs and vertebrae to approximately the anterior-posterior level of the twelfth to fifteenth vertebra (Fig. 4C; n=5). However, within the lumbar region, Nog mutants displayed reduced or absent axial cartilage. Dysmorphic condensations were present at the level of the hindlimbs. These defects were dramatically enhanced in Nog mutants additionally heterozygous for Grem1 (Fig. 4D; n=3). Within the cervical spine, both the developing pedicles and lamina were diminished. These structures were not detected within the thoracic spine, where only the proximal aspects of the anteriormost ribs were present. In addition, there were no condensations marking the primordia of the vertebral bodies at any position. We did detect weakly staining reiterating condensations of undetermined identity within the posterior axis at the level of the hindlimb, suggesting that the expression of sclerotome markers observed in the posterior of E9.5 Nog^{fx/+};Grem1^{fx/+} embryos can translate to a minimum of cartilage development.

These results prompted us to evaluate the contribution of Grem1 heterozygosity to the sclerotome defects we had observed in Nog^{fx/+};Grem1^{fx/+} animals at embryonic stages. Indeed, Pax1 expression in E9.5 Nog^{fx/+} embryos revealed larger somites that extend posteriorly through the presumptive lumbar region (Fig. 4E; n=5), when compared with Nog^{fx/+};Grem1^{fx/+} examples (Fig. 2G). Similarly, E9.5 Nog^{fx/+} Pax1 expression appeared in more posterior aspects of the body axis (Fig. 4F; n=5). Based on our expression analysis for MEO1 and Pax1, our qualitative assessment is that wherever somites form in either Nog^{fx/+};Grem1^{fx/+} or Nog^{fx/+} embryos, a proportionally sized domain of sclerotome is induced. It is only in the absence of both Nog and Grem1 that sclerotome specification and axial skeleton formation fail.

Taken together, these results show that the inactivation of even a single Grem1 allele has dramatic consequences for the Nog mutant skeletal phenotype, confirming that these antagonists interact during sclerotome development. Furthermore, we suggest that these results define the BMP antagonist-dependent stage in sclerotome development. It is known that, following induction, sclerotome cells become mesenchymal and migrate laterally, medially and dorsally and become the primordia of ribs, spinaous processes and vertebral bodies, respectively. The genetic programs directing morphogenesis of these different axial skeletal features are distinct. For example, Uncx4.1 is specifically required for lateral sclerotome-derived ribs (Leitges et al., 2000), whereas Pax1 and Pax9 cooperate in the formation of the vertebral column (Peters et al., 1999). The generalized axial skeleton agenesis observed in Nog^{fx/+};Grem1^{fx/+} animals suggests that Nog and Grem1 function early in sclerotome development prior to the subdivision of the different sclerotome compartments.
Aberrant cell death and proliferation do not explain sclerotome agenesis

We next sought to ascertain the basis of the failure to form sclerotome in Nogfx/fx; Grem1fx/tx double mutants. Either cell death, or a failure to proliferate, or a combination of these two forces early in the sclerotome lineage could explain the absence of axial skeleton progenitors. As the Nogfx/fx;Grem1fx/tx mutant phenotype is detectable by E9.0, any aberrant cell death or proliferation would have to occur prior to this stage. We dissected trunk tissue spanning somites 8-12 from embryos in the process of turning (Theiler stages 13-14). These somites are recently formed (based on 120-minute periodicity, they are between 2 and 8 hours old) and, in double mutants, represent a position at which somites exist but sclerotome markers are never observed. Sections processed for TUNEL staining from Nogfx/fx;Grem1dx/dx control and Nogfx/fx;Grem1dx/dx mutant samples did not reveal any pattern of cell death (Fig. 5A,B). The dorsal neural tube of E10.5 Nog mutant embryos exhibits high levels of apoptosis (McMahon et al., 1998) (Fig. 5C). We detected similar TUNEL-positive cells in the dorsal neural tube of Nogfx/fx;Grem1dx/dx and Nogfx/fx;Grem1dx/dx embryos at E9.0 (Fig. 5D). However, very few apoptotic cells were detected in other structures.

Although the thoracic-level somites of E8.5-9.0 Nogfx/fx;Grem1dx/dx double mutants were smaller than those of controls, there was no apparent size difference between double mutants and Nog mutants. Nevertheless, cell cycle defects within the ventral-medial somite could contribute to the sclerotome-deficient phenotype. We stained sections for phosphorylated histone H3 (PH3) to determine the rates of proliferation in control, Nog, and Nog; Grem1 double mutants. Based on morphology, we estimated the perimeter of the somite within a transverse section. To calculate proliferation rate, we expressed the number of PH3-positive nuclei per unit area of somite as a percentage of all somitic nuclei. The three genetic classes analyzed – control Nogfx/fx, Grem1dx/dx (left), Nog mutant Nogfx/fx;Grem1dx/dx (center), and double-mutant Nogfx/fx;Grem1dx/dx (right) – exhibited no significant differences in somitic proliferation rates. A minimum of three embryos of each genotype were analyzed, and at least five sections from each embryo were counted. Error bars indicate SEM.
prior to in situ hybridization. Dissection. Embryos were processed for Tamoxifen was administered via the mother at E7.5, 40 hours before expressed tamoxifen-inducible estrogen receptor fused to Cre (A–E,F). We generated males heterozygous for a antagonist gene function, we also examined sclerotome induction formation. To receive or interpret the signals necessary for sclerotome specification.

possibility that our analysis missed a critical stage, our data indicate that the failure to form sclerotome is not a consequence of early cell death or a lack of proliferation. Rather, we hypothesize that cells within the ventral somite of Nog;Grem1 mutant embryos fail to receive or interpret the signals necessary for sclerotome specificiation.

Inhibition of BMP signaling in a Nog+/− Grem1+/− background does not influence sclerotome formation

As a complement to our analysis of mutants lacking BMP antagonist gene function, we also examined sclerotome induction following interference with BMP signaling. We employed two strategies to block BMP signal transduction. First, we used conditional mutants for the type I BMP receptor Bmpr1a (Fig. 6A,B,E,F). We generated males heterozygous for a Bmpr1a null allele (Mishina et al., 1995) that also bear the ubiquitously expressed tamoxifen-inducible estrogen receptor fused to Cre recombinase [Bmpr1a+/−;Tg(Cre/Esr1)] (Hayashi et al., 2002). These animals were crossed to females homozygous for both the Bmpr1a conditional allele and the Rosa26 reporter (Bmpr1a+/−;R26R) (Mishina et al., 2002; Soriano, 1999). Tamoxifen was administered at E7.5 and animals were analyzed at E9.5. Embryos with the genotype Bmpr1a+/−;Tg(Cre/Esr1) stained strongly for β-galactosidase, suggesting high levels of Cre recombinase activity (Fig. 6B,F). However, these embryos showed no change in Pax1 expression when compared with siblings that did not express Cre (Fig. 6B; see Fig. S5 in the supplementary material). By contrast, Lbx1 expression was absent or dramatically reduced in these Bmpr1a conditional mutants (Fig. 6F; see Fig. S5 in the supplementary material). As BMP signals are involved in the induction of lateral dermomyotome derivatives (Hirsinger et al., 1997; Pourquie et al., 1996; Tonegawa et al., 1997), this observation demonstrates the efficacy of this conditional mutagenesis approach. Nevertheless, we saw no change in sclerotome development resulting from Bmpr1a conditional mutation.

To corroborate these findings, we used the drug dorsomorphin to block type I BMP receptor function in cultured whole embryos (Yu et al., 2008). Embryos were cultured from E8.5 for 30 hours in media supplemented with either 2.5 μM dorsomorphin (Fig. 6D,H) or DMSO vehicle alone (Fig. 6C,G). Control embryos generally appeared morphologically normal and expressed normal levels of Pax1 and Lbx1. Exposure to dorsomorphin did not interfere with somitogenesis, turning or neural tube closure but did result in blistering of the epidermis, associated hematomas and underdevelopment of the allantois. As in Bmpr1a conditional mutants, Pax1 was expressed normally, whereas Lbx1 expression was either diminished or lost.

These data show that the inhibition of BMP signal transduction beyond the levels normally mediated by Nog and Grem1 does not result in a bias toward sclerotome in the newly formed somite. Our findings are consistent with a model in which the function of BMP antagonists in the developing trunk is to block a BMP signal that suppresses sclerotome induction, rather than to provide an instructive signal.

Elevated BMP signaling prevents Hh-dependent sclerotome specification

The absence of BMP antagonists should result in elevated BMP signaling. To prove that the failure to form sclerotome in Nog+/−;Grem1+/− embryos is a consequence of excessive BMP signaling, we cultured double-mutant embryos from E8.5 for 30 hours in media supplemented with either 2.5 μM dorsomorphin or DMSO vehicle alone. In all cases (16/16), Pax1 expression was rescued in dorsomorphin-treated double-mutant specimens (Fig. 7A,B).

We next sought to define how elevated BMP signaling interferes with sclerotome induction. Hh signaling is required for sclerotome development (Zhang et al., 2001). However, in a previous study using explanted paraxial mesoderm, sclerotome marker expression was triggered in response to Nog even after pharmacological inhibition of Hh signaling, suggesting the possibility of an Hh-independent sclerotome induction mechanism (McMahon et al., 1998). However, we found that inhibition of BMP signaling with 2.5 μM dorsomorphin failed to rescue Pax1 expression in Smo null embryos (n=2), demonstrating that even in a BMP signaling-deficient state, Hh signaling is essential for sclerotome specification (Fig. 7B,C).

We reasoned that compromised Hh signaling within the developing somites could explain the absence of sclerotome in Nog+/−;Grem1+/− embryos. To characterize the state of Hh signaling, we compared control and mutant E9.0 expression of Shh, the Hh signaling-inhibitory receptor Ptc (Ptc1 – Mouse Genome Informatics) and the Gli transcription factors that mediate the transcription of Hh-regulated genes (Fig. 7E–G). Shh transcript was detected in all genetic classes, suggesting that Shh is produced in Nog+/−;Grem1+/− double mutants (not shown). We reasoned that Ptc and Gli1, which are directly regulated by Hh signaling (Buttitta et al., 2003; Dockter and Ordahl, 2000), would best indicate functional Hh signaling. We were surprised to observe Ptc expression in the compartment that normally becomes sclerotome (Fig. 7G, inset). Moreover, somitic expression of Gli1, Gli2 and Gli3 (Fig. 7H–M, data not shown)
...expression was elevated following Smo activation. Nog was detected in Nog\textsuperscript{fx/fx};Grem1\textsuperscript{fx/fx} embryos. We conclude that key genes that mediate Hh signal transduction are expressed and induced at relatively normal levels in the somites of double mutants.

To further test the hypothesis that sclerotome deficiency might be a consequence of defective Hh signal transduction, we again used whole-embryo culture. We treated E8.5 embryos for 30 hours in media supplemented with either 7.5 μM purmorphamine or vehicle alone. Purmorphamine activates the Hh pathway by targeting Smo (Sinha and Chen, 2006). This manipulation induced a significant increase in the expression of Ptc and Pax1 in Nog\textsuperscript{fx/fx};Grem1\textsuperscript{fx/+} control embryos, as indicated by QPCR (Fig. 7N). Likewise, Ptc expression was elevated following purmorphamine treatment in both Nog\textsuperscript{fx/fx};Grem1\textsuperscript{fx/+} and Nog\textsuperscript{fx/fx};Grem1\textsuperscript{fx/fx} samples. However, we were surprised to find Pax1 expression significantly reduced following Smo activation. In addition, we never detected somitic Pax1 expression by in situ hybridization in purmorphamine-treated Nog\textsuperscript{fx/fx};Grem1\textsuperscript{fx/fx} embryos (n=3; data not shown). The failure to respond to Smo activation strongly suggests that Hh signaling leading to Pax1 activation is defective in BMP antagonist mutants. We propose that a BMP-mediated alteration in the response to Hh signaling in the somite is the basis of sclerotome agenesis in Nog\textsuperscript{fx/fx};Grem1\textsuperscript{fx/fx} double mutants. Furthermore, these data show that BMP signaling can produce a specific switch in how the Hh signal is interpreted.

**DISCUSSION**

Our analysis shows that Nog and Grem1 cooperate to maintain a zone of reduced BMP signaling within the naïve somite that is essential for sclerotome specification. Ablation of Nog and Grem1 results in elevated BMP signaling that interferes with Hh signal transduction crucial for sclerotome induction at a step downstream of Smo. This work expands our understanding of the genetics underlying sclerotome induction, defining a developmental mechanism in which two factors secreted from different source tissues act together in pattern formation. Furthermore, our findings reveal an important example of how BMP signaling can restrict the consequences of Hh signal transduction.

**BMP blocks specification of axial skeleton progenitors**

Our findings confirm that BMP signaling must be attenuated during the specification of the cells that go on to form the axial skeleton. Once the ventral medial somite has committed to the sclerotome lineage, BMP has a positive role in the growth and elaboration of the axial skeleton (Monsoro-Burq et al., 1996; Murtaugh et al., 1999) by maintaining an Nkx3.2- and Sox9-dependent chondrogenic regulatory loop (Zeng et al., 2002). The expression of Nog in the notochord and Grem1 in the somite reflects this transition: coincident with sclerotome specification, which occurs at ~E9.0 for the thoracic region, antagonist expression in these domains begins to diminish.
Our findings are consistent with a model of sclerotome induction in which Hh is the only instructive signal required. It has been hypothesized that Nog might also direct sclerotome specification (McMahon et al., 1998). Although the work presented here does not prove that Nog cannot mediate Hh-independent sclerotome induction, we have no evidence supporting such a mechanism in vivo. Rather, our data suggest that BMP antagonists act to provide a permissive environment for sclerotome specification. Neither genetic nor pharmacological interference with BMP signaling results in a bias toward the sclerotome fate, as would be predicted if BMP antagonists were driving differentiation. Furthermore, in an embryo incapable of Hh signal transduction owing to Smo ablation, blocking BMP signaling does not restore sclerotome marker expression.

In addition to the sclerotome, the loss of Nog and Grem1 has consequences for other axial tissues. Development of the dermomyotome is also perturbed in Nog and Nog;Grem1 double mutants, as indicated by diminished Pax3 and Myf5 expression. A possible explanation for reduced expression lies in the overall smaller size of somites in these embryos. However, BMP-mediated interference with the development of dermomyotome could be consistent with our results and our understanding of early myogenesis. Hh signaling is required for efficient dermomyotome induction (Borello et al., 2006; Borycki et al., 1999; Chiang et al., 1996; Gustafsson et al., 2002; Teboul et al., 2003; Zhang et al., 2001). The defective Hh signaling that results from elevated BMP signaling may also cause defective dermomyotome development. Finally, it should be noted that although our analysis focuses on the role of BMP antagonists in somite pattern formation, we also found that excessive BMP signaling interferes with somite formation. This observation is consistent with the ability of BMP antagonists to induce somitic fates in Xenopus explants that are specified as ventral mesoderm, and to induce ectopic somites in chick lateral plate (Smith et al., 1993; Tonegawa et al., 1997). How BMP interferes with the somite formation process is not known.

**BMP interferes with Hh target transactivation**

Defining how signaling pathways interact is crucial for understanding cell fate decisions. Our analysis shows that, within the somite, BMP signaling results in selected disruption of Hh signaling, as indicated by the failure of Smo activation to induce expression of the sclerotome marker Pax1 in Nog\textsuperscript{fx/fx};Grem1\textsuperscript{f/c/c} embryos. As we have detected transcripts of components of the Hh signaling pathway, including the transcription factors Gli2 and Gli3 that are required for sclerotome (Buttitta et al., 2003), we hypothesize that elevated BMP signaling results in the production of a dominant repressor of Pax1 expression in Nog;Grem1 double mutants. We argue that this effect is selective, as Hh signal transduction and activation of gene expression are still effective in activating Ptc. The surprising reduction in Pax1 expression elicited by purmorphamine in Nog\textsuperscript{f/c/c};Grem1\textsuperscript{f/c/c} and Nog\textsuperscript{f/c/c};Grem1\textsuperscript{f/c/c} embryos suggests that the context of BMP activation converts the activating Hh signal to a repressive one. Alternatively, BMP leads to a dominant repressive activity on the Pax1 promoter.

A mechanism in which BMP signaling functions in opposition to Shh signaling is not without precedent. In the ventral neural tube, which is patterned by notochord- and floor plate-derived Shh, BMPs induce more dorsal cell types, although in this context the dose of BMP is instructive (Liem et al., 2000; Timmer et al., 2002). Conversely, zebrafish bearing mutations in BMP pathway components exhibit a ventralized neural tube (Barth et al., 1999). In cerebellar granule neuron progenitors and primary medulloblastoma cells, Shh-dependent proliferation is blocked by BMP-induced degradation of the Hh target Atoh1 (Math1) (Zhao et al., 2008). It will be important to define the differences between the transactivation of contextual targets of Hh signaling, such as Pax1 in the sclerotome, and invariant targets, such as Ptc, in different Hh-responsive cell types as well as how other developmental pathways affect the transcriptional readout. As a general word of caution, this work also demonstrates that active Ptc expression does not necessarily imply that the Hh signal transduction leading to other Hh targets is functioning normally.

**Nog and Grem1 cooperate despite derivation from different sources**

Nog and Grem1 are not the only BMP antagonists expressed in the embryonic trunk. Chrd is expressed in the node and later in the notochord and follistatin is expressed in the PSM and the developing somites. It might be significant that approximately one-third of Nog;Grem1 double mutants exhibit faint expression of Pax1 in anterior somites. This limited sclerotome induction could be a result of compensation from additional BMP antagonists. Notably, the expression domains of Nog and Grem1 do not overlap. We suggest that the cooperative function of signals secreted from different source tissues might be a common feature of developing systems. With regard to the developing skeleton and the signaling pathways involved, a wide array of genes with different expression domains but overlapping functions might have been a significant factor in generating diversity in skeletal forms.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.051938/-/DC1

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