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A critical role for sFRP proteins in maintaining caudal neural tube closure in mice via inhibition of BMP signaling

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ABSTRACT

Both the BMP and Wnt pathways have been implicated in directing aspects of dorsal neural tube closure and cell fate specification. However, the mechanisms that control the diverse responses to these signals are poorly understood. In this study, we provide genetic and functional evidence that the secreted sFRP1 and sFRP2 proteins, which have been primarily implicated as negative regulators of Wnt signaling, can also antagonize BMP signaling in the caudal neural tube and that this function is critical to maintain proper neural tube closure and dorsal cell fate segregation. Our studies thus reveal a novel role for specific sFRP proteins in balancing the response of cells to two critical extracellular signaling pathways.

Introduction

The proper development of all multicellular organisms requires the spatial and temporal coordination of numerous extracellular signaling cues. Developing embryos comprise a complex and dynamic environment where cells are typically exposed to many signals that may serve either synergistic or antagonistic roles. A key question is how cells within such an environment regulate their responses to these competing influences to enact appropriate cell fate specification and differentiation programs.

In vertebrates, five *sFRP* genes have been identified, each showing dynamic and partially overlapping expression patterns in many tissues during development and in adults (Chapman et al., 2004; Esteve et al., 2000; Leimeister et al., 1998; Terry et al., 2000). These genes encode a family of secreted proteins that share homology with the Wnt Frizzled (Fz) receptors and can competitively bind Wnt ligands and were initially identified as negative extracellular regulators of Wnt signaling (Bovolenta et al., 2008; Jones and Jomary, 2002; Kawano and Kypta, 2003; Rattner et al., 1997). In the CNS, both sFRP1 and sFRP2 have been shown to be capable of inhibiting canonical Wnt signaling (Galli et al., 2006; Lopez-Rios et al., 2008; Rodriguez et al., 2005). In addition, mutations in *sFRP* genes are associated with a number of Wnt-related cancers and similar disorders, consistent with their role as negative regulators of this pathway (Shulewitz et al., 2006). However, the close sequence and structural similarity of these proteins and the possibility of redundant function has hampered a clear definition of their individual and overlapping roles in vertebrate embryogenesis.

Recently, studies in frog and zebrafish embryos have implicated sFRP family proteins as mediators of BMP signaling during embryonic axis formation, a classic model system for identifying and assaying BMP antagonist activities (Lee et al., 2006; Muraoka et al., 2006). In these studies, it was shown that sizzled, the anamniotic sFRP homolog, could block BMP signaling by binding to and inhibiting the activity of Tolloid/BMP1, a metalloproteinase that positively regulates Bmp signaling by cleaving and inactivating the extracellular BMP inhibitor Chd. Notably, the mouse sFRP2 protein was also found to possess Tolloid/BMP1 inhibitory activity, suggesting that aspects of this interaction or function could be conserved across vertebrate species. Together, these data raise the possibility that vertebrate sFRP proteins have the potential to simultaneously regulate both Wnt and BMP signaling and therefore coordinate their activities, although experimental evidence supporting such a role in vivo has not yet been provided.

In this study, we present evidence that sFRP1 and sFRP2 play critical roles during vertebrate development by regulating BMP signaling in the dorsal neural tube and ectoderm. We show that mouse *sFRP1;sFRP2* double mutants exhibit a unique neural tube defect (NTD) phenotype in the caudal spinal cord that is associated with an up-regulation of both BMP and Wnt signaling in this tissue. Using a gain-of-function assay, we furthermore demonstrate that many aspects of the mouse *sFRP1;sFRP2* mutant NTD phenotype can be experimentally modeled in chicks by BMP4 overexpression and that sFRP1 and sFRP2 can specifically inhibit these effects by blocking

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BMP signaling independent of their effect on Wnt signaling. Our study suggests a new role for sFRP proteins as critical coordinators of BMP and Wnt signals during vertebrate embryogenesis.

Materials and methods

Animals

Targeted mouse *sFRP1* (Bodine et al., 2007) and *sFRP2* (Kobayashi et al., 2009) mutants were maintained on an outbred SW background. For staging litters, the morning of plug detection was designated 0.5 days postcoitus (dpc). To obtain litters containing double-mutant embryos, *sFRP1*$^{+/-}$;*sFRP2*$^{+/-}$ adults were intercrossed, and embryos were genotyped using PCR as described (Bodine et al., 2007; Lei et al., 2006). Fertilized White-Leghorn chicken eggs were obtained from Charles River Labs and stored at room temperature until being placed in an incubator at 38 °C. Electroporations were performed as described (Lei et al., 2004).

Plasmids and constructs

chd, noggin, sFRP1, sFRP2, and Dkk1 cloned into the bi-cistronic pCIG vector that also encodes GFP.

For cotransfection experiments, mouse BMP4 was subcloned into the pCS2 vector (which does not encode GFP). Transfection efficiency was monitored in adjacent sections using RNA in situ hybridization with a probe that is specific for mouse, but not chick, BMP4, while GFP expression was used to monitor the expression of the cotransfected cDNA.

Immunohistochemistry and mRNA in situ hybridization

These procedures were performed as described (Lei et al., 2004).

Results

Failure to maintain caudal neural tube closure in sFRP1;sFRP2 double-mutant embryos

To define the unique and overlapping roles of *sFRP1* and *sFRP2* in CNS development, we analyzed single and compound mutant embryos from targeted *sFRP1* and *sFRP2* mouse lines (Bodine et al., 2004; Kobayashi et al., 2009; Lei et al., 2006). It was previously reported that double homozygous embryos exhibit somite patterning defects in thoracic regions and a truncated tail at early embryonic stages (Satoh et al., 2006; Satoh et al., 2008). We also observed a neural tube defect (NTD) localized to the lumbar region caudal to the hind limbs in nearly 100% of the *sFRP1*$^{-/-}$;*sFRP2*$^{-/-}$ double homozygous (DKO) mutant embryos examined at embryonic day (E) 10.5 and 11.5 (Figs. 1a–c; $n = 23/25$ embryos examined; data not shown) that bore a striking similarity to a human condition known as hydromelia (Ikenouchi et al., 2002). Examination of sections through the affected region at E10.5 revealed an apparent enlargement of the dorsal neural tube (Figs. 1d and e), which became highly disrupted at E11.5 (Fig. 1f). Staining with antibodies to E-cadherin to mark the non-neural ectoderm (NNE) showed that a contiguous epithelium was present over the expanded dorsal neural tube at E10.5, which impinged into the central canal at E11.5 (Figs. 1g–i and o). Notably, the NNE overlying the neural tube was thickened in double mutants compared to wild-type (*WT*) embryos beginning at E10.5 and became progressively thicker by E13.5, even in thoracic regions where no NTD was evident (Figs. 1g–i; Fig. S1). No dorsal neural tube defects were found in either single mutant or in double mutants containing at least 1 normal allele of either *sFRP1* or *sFRP2* (Figs. 1n and q; data not shown).

To further characterize the origin and progression of the NTD, we collected embryos at stages between E10.5 and E13.5 and stained tissue sections with markers that identify specific cell types. In DKO embryos at E10.5, the roof plate (RP) marker Lmx1a (Millonig et al., 2000) was detected in a broader domain than in either wild-type (*WT*), *sFRP1*$^{-/-}$, or *sFRP2*$^{-/-}$ single mutants (Figs. 1m–o; data not shown). Also at this stage, the BrdU incorporation rate for cells in the enlarged dorsal midline region was similar to the RP in *WT* embryos in which short pulse times (≤30 minutes prior to sacrifice) labeled fewer cells than in adjacent areas where the neuroepithelium is proliferating more rapidly (Figs. 1j and k). In addition, the expression of activated caspase-3, a marker for cells undergoing apoptosis, was sharply elevated in dorsal midline cells in DKO embryos (Figs. 1p–r). Together, this analysis reveals that the lumbar NTD in *sFRP1;sFRP2* double homozygotes arises from an abnormal expansion of dorsal midline and NNE tissues after neural tube closure accompanied by elevated cell death in the dorsal midline of the spinal cord, leading to the collapse of the NNE into the central canal.

The unique NTD phenotype in *sFRP1;sFRP2* double homozygous mutant embryos compared to either single mutant alone prompted us to carefully examine the expression patterns of these two genes in the lumbar region around the time of caudal neural tube closure (E8.0–9.5 in the developing mouse). At late neural plate/neural fold stages (E8–8.5), *sFRP1* transcripts were detected in a continuous band of cells at the margin of the neural plate and NNE, as well as in a medial (future ventral) region of the neuroepithelium, but no expression was seen in the dorsal neural folds (Fig. 2a). This basic pattern persisted after neural tube closure, with *sFRP1* expression being detected in the ependymal zone cell layer lining the central canal, and NNE throughout neurogenesis (Figs. 2b and c). In contrast, *sFRP2* expression at E8.5 was not detected in the NNE, but instead was widely expressed in the neurectoderm (Lei et al., 2006), with strong expression in the dorsal neural folds (Figs. 2d and g). Between E9.5 and 10.5, *sFRP2* expression became progressively restricted to two domains flanking the dorsal midline in lower thoracic and lumbar regions, concomitant with the induction of the RP (Chizhikov and Millen, 2004b) as well as to the ventral neural tube (Figs. 2e, f, h, i, and w). Analysis of *sFRP1* and *sFRP2* expression in single mutants showed that neither gene requires the other for its expression (Figs. 2m–r). Thus, *sFRP1* and *sFRP2* are expressed in adjacent and largely non-overlapping dorsal tissues in the lumbar region during neural tube closure stages.

The complementary expression patterns of *sFRP1* and *sFRP2* in dorsal regions, taken with the observation that the NTD does not arise unless both genes are absent, suggest the possibility that these factors may independently regulate signalling between the neural tube and NNE following neural tube closure. To examine this possibility, we studied the expression of *BMP4* and *Wnt6*, two factors expressed in the NNE that have been shown to play a role in providing important cell fate cues in these tissues (Garcia-Castro et al., 2002). Notably, we found that *BMP4* expression was up-regulated in dorsal NNE overlying the neural tube in *sFRP1*$^{-/-}$ and DKO embryos compared to *WT* or *sFRP2* mutants (Figs. 2s–v). In contrast, *Wnt6* expression in the NNE was unaltered in *sFRP1* and *sFRP2* mutants but was down-regulated in DKO embryos (data not shown). Together, these observations show that loss of *sFRP1* alone is sufficient to give rise to increased BMP4 expression from the NNE but not for the development of the NTD, which requires the additional loss of *sFRP2* from the neural tube.

Incomplete segregation of neural crest and roof plate fates in sFRP1;sFRP2 double-mutant embryos

The nature of the dorsal neural tube defect in *sFRP1;sFRP2* double-mutant embryos prompted us to study the development of cells that normally derive from this region. To do this, we used antibodies against key transcription factors that identify distinct cell types in mutant embryos at E9.5–10.5. Neural crest (NC) cells are induced at neural fold stages by signals emanating from the adjacent NNE and