Conserved function of *mSpry-2*, a murine homolog of *Drosophila sprouty*, which negatively modulates respiratory organogenesis

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In *Drosophila* embryos, the loss of *sprouty* gene function enhances branching of the respiratory system. Three human *sprouty* homologues (*h-Spry1–3*) have been cloned recently, but their function is as yet unknown [1]. Here, we show that a murine *sprouty* gene (*mSpry-2*), the product of which shares 97% homology with the respective human protein, is expressed in the embryonic murine lung. We used an antisense oligonucleotide strategy to reduce expression of *mSpry-2* by 96%, as measured by competitive reverse transcriptase PCR, in E11.5 murine embryonic lungs cultured for 4 days [2]. Morphologically, the decrease in *mSpry-2* expression resulted in a 72% increase in embryonic murine lung branching morphogenesis as well as a significant increase in expression of the lung epithelial marker genes *SP-C*, *SP-B* and *SP-A*. These results support a striking conservation of function between the *Drosophila* and mammalian *sprouty* gene families to negatively modulate respiratory organogenesis.

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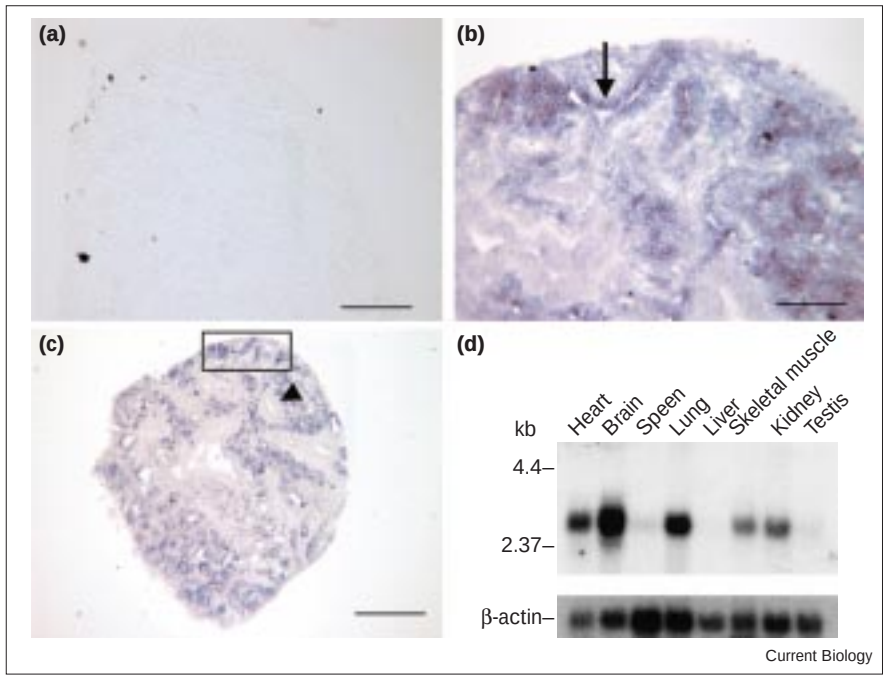
Results and discussion

The *Drosophila* respiratory system and the mammalian lung are both formed by a process of branching morphogenesis, which depends on epithelial and mesenchymal interactions mediated by signaling between members of the fibroblast growth factor (FGF) family and their cognate receptors. Branchless, a *Drosophila* FGF homologue, is expressed in the tips of tracheal branches [3]. Branchless activates an FGF receptor homologue termed Breathless [4], which directs tracheal cell migration as well as inducing secondary and terminal branches.

Figure 1

Sequence homology of mSpry-2 with the *Drosophila* and human *sprouty* gene products (h-Spry2 and d-Spry, respectively). Degenerate PCR was used to clone *mSpry-2* from E12 mouse lung cDNA resulting in a 948 bp sequence, the product of which has 97% homology to that of the *h-Spry2* cDNA sequence. (a) Amino-acid alignment of mSpry-2 with h-Spry2 and d-Spry. The numbering and alignment of amino acids is relative to the cloned sequence of *mSpry-2* and begins with the translational start site. Dashes indicate no corresponding amino acid at that position. Shaded residues indicate identical residues. The asterisk indicates amino acids that are missing when *mSpry-2* is compared with *Drosophila sprouty* cDNA sequences. K indicates a lysine residue at position 158 of the murine sequence that is not present in the other sequences. (b) Alignment of the mSpry-2 antisense and sense oligodeoxynucleotides with h-Spry2 and d-Spry sense oligodeoxynucleotides. The asterisk indicates the translational start site.

Figure 2



Localization of *mSpry-2* expression in the mouse lung. *In situ* hybridization was performed on E12 sectioned lungs as previously described [10], with minor modifications to embryonic tissue. **(a)** E12 lungs hybridized with a sense *mSpry-2* cRNA probe showed no staining within the epithelium or mesenchyme. **(b,c)** Lungs hybridized with an antisense *mSpry-2* cRNA probe displayed localized *mSpry-2* expression within the epithelial cells, predominantly in the smaller distal airways as opposed to the larger proximal airways. Panel (b) is an enlargement of the boxed region in (c). The arrow in (b) indicates the location of *mSpry-2* mRNA expression in the branch point between two terminal epithelial branches in the periphery of the lung. The arrowhead in (c) indicates *mSpry-2* mRNA expression in the airway of the lung. The scale bar represents 25 μm for (a,b) and 100 μm for (c). Magnification is 560× for (a,b) and 141× for (c). **(d)** A northern blot of RNA from multiple adult mouse tissues was hybridized with ^{32}P-labeled *mSpry-2* cDNA. A single band of RNA was identified with high levels of expression in the brain and lung and minimal abundance in the heart, skeletal muscle and kidney. RNA size markers are indicated to the left. β-actin gene expression was determined as a control. Northern analysis was performed as previously described [11].

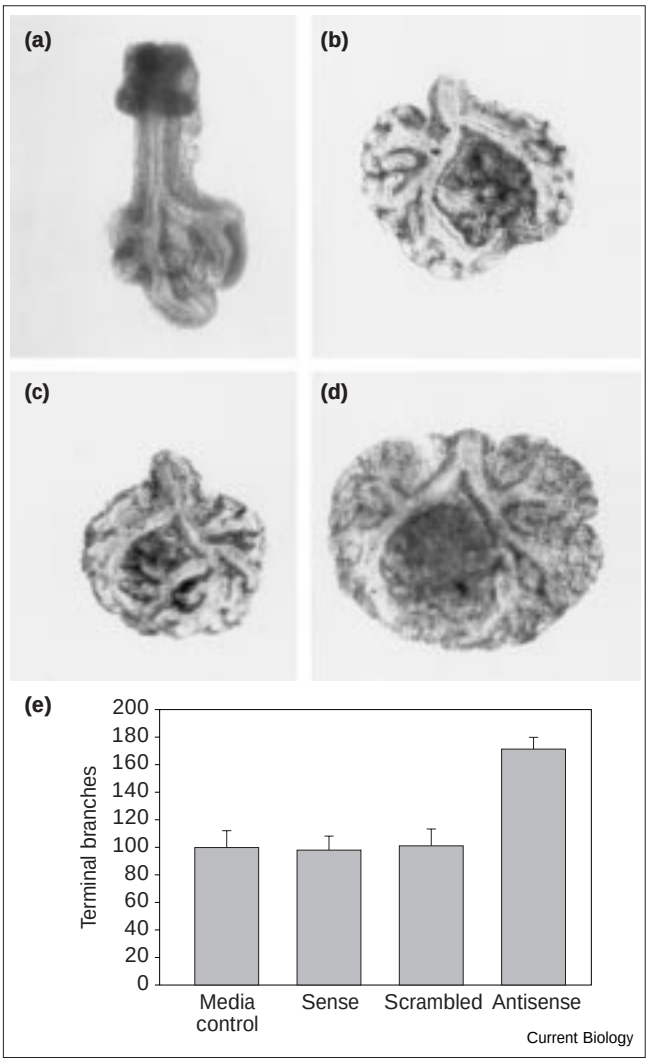
The *sprouty* gene product functions as an FGF antagonist in *Drosophila*: overexpression of Sprouty blocks activation of downstream effectors in the *Branchless* pathway, whereas *sprouty* null mutation enhances the function of *Branchless* downstream genes, resulting in enhanced tracheal branching [1]. The *Drosophila sprouty* gene encodes a 63 kDa polypeptide with a cysteine-rich region that is highly conserved in three human *sprouty* homologs (*h-Spry1–3*). The h-Spry2 amino-acid sequence is 51% homologous in the cysteine-rich region to *Drosophila* Sprouty. We designed degenerate primers to *h-Spry2* and used PCR to clone the *mSpry-2* sequence from E12 mouse lung cDNA. We found the mSpry-2 amino-acid sequence to be 97% homologous to h-Spry2 (Figure 1).

In *Drosophila*, *sprouty* expression is induced near the tracheal epithelium and restricts bud formation by inhibiting the *Branchless* pathway [1]. We found the expression of *mSpry-2* to be highly localized in embryonic lung epithelial cells, primarily in the distal airways (Figure 2a–c), suggesting that this gene may function as a spatial regulator of new bud formation. Northern analysis showed *mSpry-2* to be expressed in the adult lung as well as the heart, brain, skeletal muscle and kidney (Figure 2d).

Null mutation of the *Drosophila sprouty* gene results in enhanced tracheal branching [1], so we designed an antisense oligonucleotide to the translational start site of *mSpry-2* (Figure 1b) to determine its effects on murine lung branching morphogenesis. E11.5 lungs cultured for 4 days in the presence of mSpry-2 antisense oligonucleotide were more extensively branched than control lungs (Figure 3a–d). In the presence of 40 μM mSpry-2 antisense oligonucleotide, terminal branches increased by 72% compared with lungs grown in control media, 40 μM sense oligonucleotide, or 40 μM scrambled oligonucleotide (Figure 3e). The proliferating cell nuclear antigen (PCNA) index also increased by 30% in lungs cultured in 40 μM mSpry-2 antisense oligonucleotide ($p < 0.01$), providing evidence that some stimulation of cell proliferation also occurred following abrogation of endogenous *mSpry-2*. Competitive PCR methodology [2] showed that the increase in terminal branches was accompanied by a 96% decrease in endogenous *mSpry-2* mRNA expression (Figure 4a), compared with levels in lungs cultured with scrambled and sense oligonucleotide controls (Figure 4b). We therefore conclude that mSpry-2 antisense oligonucleotide effectively abrogated *mSpry-2* mRNA expression. This suggests that in the organization of the mammalian respiratory system, *mSpry-2* functions in a similar fashion to *sprouty* in the respiratory system of *Drosophila*.

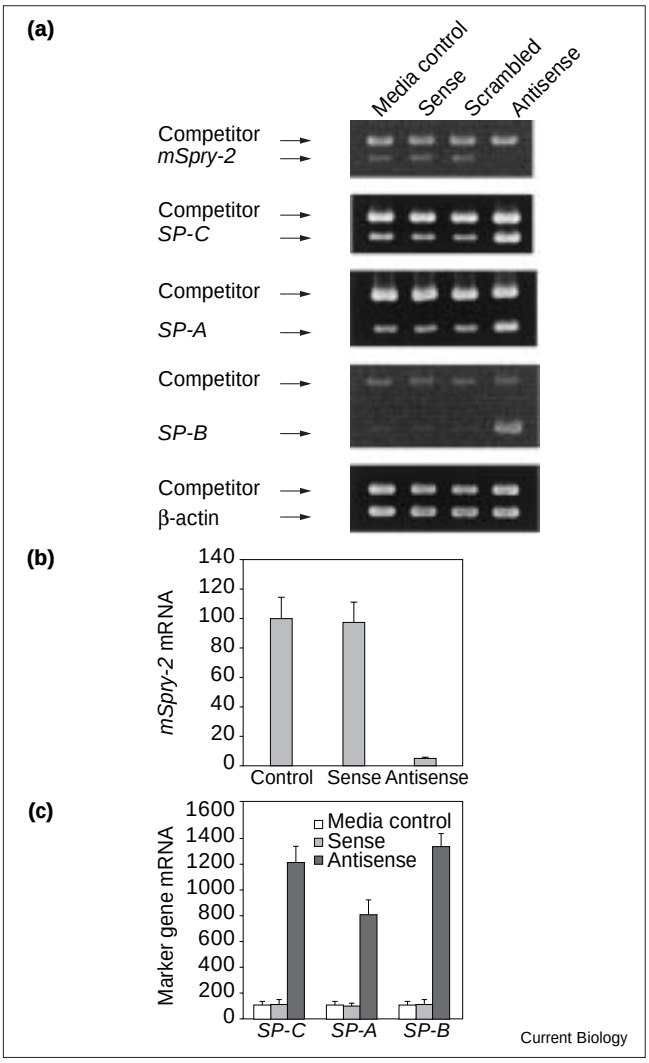
Fetal lung maturation is associated with increased expression of surfactant proteins, which are vital for effective gas exchange at birth [5]. In the developing lung, *SP-B* and *SP-A* are expressed in alveolar type 2 (AEC2) cells as well as other epithelial cells in the conducting airways, whereas *SP-C* is exclusively expressed by AEC2 cells and

Figure 3



Antisense mSpry-2 oligonucleotide enhances murine embryonic lung branching morphogenesis. (a) E11.5 lung buds were grown in chemically defined, serum-free media for 4 days (b). A 72% increase in terminal branches was observed with lungs cultured in (d) 40 μM mSpry-2 antisense oligonucleotide, compared with (c) sense oligonucleotide controls. (e) Terminal branches were quantified, showing that mSpry-2 antisense oligonucleotide significantly increased lung branching morphogenesis when compared to media controls (set at 100%) and sense and scrambled oligonucleotide controls ($p < 0.05$ in analysis of variance (ANOVA)).

Figure 4



Antisense mSpry-2 oligonucleotide decreased endogenous *mSpry-2* mRNA levels and increased *SP-C*, *SP-A* and *SP-B* mRNA levels. (a) Lungs cultured with antisense mSpry-2 oligonucleotide had reduced endogenous *mSpry-2* mRNA levels when compared with media controls and sense and scrambled oligonucleotide controls, as shown by competitive PCR methodology (described previously [10]). Abrogation of endogenous *mSpry-2* also resulted in an increase in *SP-C*, *SP-A* and *SP-B* mRNA levels. Quantitation of competitive reverse transcriptase PCR data were derived [10] and normalized to β-actin. (b) Antisense mSpry-2 oligonucleotide significantly decreased endogenous *mSpry-2* levels by 96% and (c) increased expression of *SP-C*, *SP-A* and *SP-B* mRNAs by 12-fold, 8-fold and 13-fold respectively ($p < 0.05$, ANOVA). Values are expressed as the mean ± standard deviation with the media control set at 100%.

is a specific marker for AEC2 differentiation [6,7]. *SP-C* expression can be detected as early as E11, but becomes restricted to the distal portion of the developing lung later in development, whereas *SP-A* is expressed later in gestation. In this study, these surfactant protein mRNAs were examined as epithelial-specific maturation/differentiation markers. We found that abrogation of *mSpry-2* expression resulted in a significant increase in expression of *SP-C*, *SP-A* and *SP-B* mRNAs (Figure 4a,c), which correlated with increased terminal branching. This suggests that the observed increase in branching is also associated with induction of epithelial maturation.

We suggest that mSpry-2 is a negative regulator of FGF signaling and its apparent function may be to negatively

modulate new bud formation. In the developing lung, FGF-10 is highly expressed in the distal mesenchyme and is involved in directing the outgrowth of epithelial buds [8,9]. FGF-10 expression is downregulated by Sonic hedgehog in the lung endoderm, demonstrating that this specific interaction between the mesenchyme and epithelium is critical in the regulation of lung branching morphogenesis [8]. Also, FGF-10 exerts a chemoattractant effect on embryonic lung epithelia and this effect is similar to that produced by Branchless in the *Drosophila* tracheal system [9]. We propose that in the developing mouse lung, mSpry-2 negatively modulates bud formation. Further investigation will determine possible interactions between mSpry-2 and FGF signaling.

In conclusion, we report a function for a novel FGF antagonist in mice, encoded by murine *Spry-2*, which is 97% homologous to the human *Spry-2* gene product. The *mSpry-2* mRNA is highly abundant in adult mouse lung and brain. *In situ* hybridization studies on E12 lung sections showed *mSpry-2* to be expressed within the distal epithelium. Reducing *mSpry-2* expression resulted in increased terminal branching and cell proliferation of murine embryonic lungs in culture as well as increased expression of maturation-specific surfactant-associated protein genes. This evidence supports an evolutionary conservation of function for the *sprouty* gene family in respiratory organogenesis between *Drosophila* and mouse.

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