Development 126, 4465-4475 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV3066

Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed

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Accepted 3 August; published on WWW 27 September 1999

Summary

The *Drosophila sprouty* gene encodes an antagonist of FGF and EGF signaling whose expression is induced by the signaling pathways that it inhibits. Here we describe a family of vertebrate Sprouty homologs and demonstrate that the regulatory relationship with FGF pathways has been conserved. In both mouse and chick embryos, Sprouty genes are expressed in intimate association with FGF signaling centers. Gain- and loss-of-function experiments demonstrate that FGF signaling induces Sprouty gene expression in various tissues. Sprouty overexpression obtained by infecting the prospective wing territory of the chick embryo with a retrovirus containing a mouse Sprouty gene causes a reduction in limb bud outgrowth and other effects consistent with reduced FGF signaling from the apical ectodermal ridge. At later stages of development in the infected limbs there was a dramatic reduction in skeletal element length due to an inhibition of chondrocyte differentiation. The results provide evidence that vertebrate Sprouty proteins function as FGF-induced feedback inhibitors, and suggest a possible role for Sprouty genes in the pathogenesis of specific human chondrodysplasias caused by activating mutations in *Fgfr3*.

Key words: Chondrodysplasia, FGF8, FGF signaling, Limb development, Sprouty, *Drosophila*

INTRODUCTION

Fibroblast Growth Factors (FGFs) are vital intercellular signaling molecules that regulate numerous processes (reviewed by Basilico and Moscatelli, 1992; Szebenyi and Fallon, 1999). They signal by binding to and activating specific transmembrane receptor tyrosine kinases (reviewed by Johnson and Williams, 1993). At least 18 different FGF and 4 FGF receptor (FGFR) genes are known in vertebrate species (reviewed by Coulier et al., 1997; see Ohbayashi et al., 1998 and references therein). Genetic studies in the mouse have demonstrated that FGF signaling is required for cell proliferation/survival at the time of embryo implantation (embryonic day [E] 4.0; Feldman et al., 1995; Arman et al., 1998) and for cell migration during gastrulation (beginning at ~E6.5; Ciruna et al., 1997; Sun et al., 1999). At later stages of embryogenesis FGFs regulate development of the brain, limb, lung, tooth and many other organs (reviewed by Goldfarb, 1996; Szebenyi and Fallon, 1999). FGF and FGFR genes have also been identified in *Drosophila*, and are required for migration of mesoderm and glial cells and for the control of branching events in the tracheal system (reviewed by Skaer, 1997). In worms, FGF signaling is required for migration of sex myoblasts (reviewed by Chen and Stern, 1998).

FGF signaling has been extensively studied in the vertebrate limb, where it is thought to provide the signals that induce limb formation and maintain initial outgrowth of the limb bud (reviewed by Martin, 1998). Subsequent outgrowth of the bud and specification of the limb skeletal elements is thought to depend on FGFs produced by cells in a specialized epithelium at the limb bud distal tip, known as the apical ectodermal ridge (AER). This conclusion is based on the observation that surgical removal of the AER results in limb truncation (Saunders, 1948; Summerbell, 1974), but an FGF-bead can substitute for it to provide all of the AER-derived signals necessary for limb development (Niswander et al., 1993; Fallon et al., 1994).

The AER regresses after the precursors of the skeletal elements have been specified, but FGF signaling continues to play a role in limb skeletogenesis. Mice with loss-of-function mutations in *Fgfr3* have abnormally long limb bones (Colvin et al., 1996; Deng et al., 1996), whereas mice overexpressing *Fgf2* have short limb bones (Coffin et al., 1995), and both

phenotypes are due to abnormalities in the growth plate of the skeletal elements. In addition, overexpression of *Fgf8* in chick embryos causes a complex phenotype, including short limb skeletal elements (Vogel et al., 1996). Furthermore, several of the most common human dwarfism syndromes are due to dominant activating mutations in *Fgfr3* (reviewed by Webster and Donoghue, 1997; Burke et al., 1998). Together these data indicate that FGF signaling controls the rate of endochondral ossification, and thereby determines the length of the skeletal elements.

In view of the importance of FGF signaling in limb formation and many other developmental processes, it was intriguing when a gene that modulates FGF signaling was identified in a screen for mutations that affect tracheal branching in *Drosophila* (Hacohen et al., 1998). In wild-type embryos, FGF signaling guides outgrowth of primary tracheal branches and induces cells closest to the FGF signaling center to form secondary branches (Sutherland et al., 1996). In sprouty (*spry)* null mutants, the FGF pathway is overactive and ectopic secondary branches are induced in cells farther from the FGF signaling center. Overexpression of *spry* during primary branch outgrowth causes the opposite effect, inhibiting the FGF inductive pathway and blocking all secondary branching. Thus, *spry* antagonizes FGF signaling in the developing trachea. Importantly, expression of *spry* is induced by FGF signaling. Therefore, the FGF pathway regulates the expression of its own antagonist, thereby limiting the range over which FGF signaling is active. Recently, *spry* was found to inhibit EGF and possibly other RTK signaling pathways (Bergmann et al., 1998; Casci et al., 1999; Kramer et al., 1999).

spry encodes a novel protein with a unique cysteine-rich domain, which localizes to cell membranes (Hacohen et al., 1998; Casci et al., 1999), but the biochemical mechanism by which it antagonizes FGF and other signaling pathways is not fully understood. Studies in cultured *Drosophila* cells and in vitro binding experiments have led to the proposal that it is localized at the inner surface of the plasma membrane and antagonizes RTK signaling pathways by direct binding of the signal transduction components Gap1 and the Grb2 homolog Drk (Casci et al., 1999). However, endogenous SPRY protein in developing wing discs is found in punctate structures that resemble secretory vesicles or endocytic uptake vesicles, and it has also been suggested to function as an extracellular antagonist to explain its cell non-autonomous actions in the tracheal system (Hacohen et al., 1998; Kramer et al., 1999).

Three human genes have been identified with sequence similarity to *Drosophila spry* (Hacohen et al., 1998), and a fourth family member has been identified in mouse (de Maximy et al., 1999). Here we have investigated the relationship between Sprouty genes and FGF pathways in vertebrates, and have begun to explore Sprouty gene function in vertebrate embryogenesis. Our data indicate that FGF signaling regulates expression of Sprouty genes in the mouse and chick embryo, and suggest that in turn, Sprouty gene expression antagonizes FGF signaling. In addition, we show that overexpression of Sprouty genes in the chick limb leads to inhibition of chondrocyte differentiation that results in a chondrodysplasia resembling that observed in individuals with activating mutations in *Fgfr3*. This raises the possibility that induction of Sprouty gene expression plays a role in these human skeletal malformation syndromes.

MATERIALS AND METHODS

Cloning and chromosome mapping

Fragments of the cysteine-rich domains of human (h)*Spry1*, h*Spry2*, h*Spry3* and mouse (m)*Spry4* were amplified by PCR using primers derived from EST sequences and either h*Spry1* and h*Spry2* EST plasmids or mouse genomic DNA as template. The amplification products were used to screen a neonatal mouse brain cDNA library in the λZAP vector (Stratagene; La Jolla, CA) for m*Spry1*, m*Spry3* and m*Spry4*, an E11 mouse embryo cDNA library (Clontech; Palo Alto, CA) for m*Spry2*, a stage 14-16 chick embryo cDNA library (kindly provided by C. Tabin) for chick (c)*Spry1*, and an E10 chick embryonic brain cDNA library (kindly provided by T. Kennedy and M. Tessier-Lavigne) for c*Spry2*. Hybridizations were performed at 55°C for 16 hours (1% BSA, 0.2 M sodium phosphate, 15% formamide, 1 mM EDTA, 7% SDS), followed by three 20 minute washes at 55°C in 2× SSC/1% SDS, and three 20 minute washes at 55°C in 0.2× SSC/0.1% SDS. For m*Spry1* both strands of a 2.5 kb clone representing the major transcript were sequenced. For m*Spry2* and m*Spry4* both strands of a single clone containing the complete coding region were sequenced. The c*Spry2* sequence was compiled from three overlapping partial cDNAs. GenBank accession numbers: mSpry1, AF176903; mSpry2, AF176905; mSpry4, AF176906; cSpry1, AF177875; cSpry2, AF176904.

Chromosome mapping was performed using The Jackson Laboratory (Bar Harbor, ME) interspecific backcross panels BSB ([C57BL/6J × M. spretus]F_1 × C57BL/6J), and BSS([C57BL/6JEi × SPRET/Ei]F_1 × SPRET/Ei) (Rowe et al., 1994). Restriction site polymorphisms were identified between parental strains. The backcross panels were genotyped and the data were submitted to the Jackson Laboratory for analysis. The data are available at www.jax.org/resources/documents/cmdata.

RNA in situ hybridization, BrdU labeling, skeletal preparations and histology

Embryos were obtained by mating CD1 mice (Charles River Laboratories; Hollister, CA) or mice heterozygous for an *Fgf8* null (–) allele, $Fgf8^{\Delta 2,3n}$ (Meyers et al., 1998). Noon of the day of plug detection was considered E0.5. $Fgf8^{-/-}$ embryos were identified by their characteristic morphology (Sun et al., 1999). White Leghorn chick embryos (California Golden Eggs; Sacramento, CA), were staged by the criteria of Hamburger and Hamilton (1951). Whole-mount and paraffin section in situ hybridization analyses were performed as previously described (Neubüser et al., 1997). Probes were prepared from plasmids containing the complete protein coding region and 3′UTR (m*Spry1*, m*Spry2*, m*Spry4*) or sequences extending 5′ from the 3′UTR into the region encoding the cysteine-rich domain (m*Spry3*, c*Spry1*, c*Spry2*). The cDNA sequences used as probes for mouse and chick *Fgf8* were described previously (Crossley and Martin, 1995; Crossley et al., 1996b) and the chick *Sox9* probe was kindly provided by C. Tabin. BrdU labeling and analysis were performed as described by Zou et al. (1997).

For cartilage staining, embryos were fixed overnight in 5% trichloroacetic acid, rinsed in water, stained with Alcian blue overnight, washed in acid alcohol (75% ethanol, 25% glacial acetic acid), then 100% ethanol, and cleared in methyl salicylate. Combined bone and cartilage staining was performed essentially as described by Otto et al. (1997). For histology, embryos were fixed in 4% paraformaldehyde/PBS, decalcified in Cal-Ex (Fisher; Santa Clara, CA), embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E).

Bead implantation, retrovirus construction and infection protocol

FGF-beads were prepared as previously described (Crossley et al., 1996b), using hFGF2 (~0.5 mg/ml, GIBCO-BRL; Grand Island, NY), hFGF4 (~1.0 mg/ml, kindly provided by Genetics Institute; Cambridge, MA) or mFGF8 (~0.8 mg/ml, kindly provided by C. MacArthur). Implantation of beads into mouse mandibular arch explants and chick embryos in ovo were performed as previously described by Neubüser et al. (1997) and Crossley et al. (1996a,b), respectively.

m*Spry2* and m*Spry4* cDNAs spanning the entire coding region were subcloned into the retroviral vector RCAS(BP). GFP virus was kindly provided by C. Tabin. Virus was produced, concentrated, titered and injected essentially as described by Logan and Tabin (1998). Pathogen-free fertilized chicken eggs were obtained from SPAFAS (Storrs, CT). The titer of injected virus was ~10^8 IU/ml. The efficiency of infection and extent of transgene expression was assessed ~24-30 hours after infection (at stages 17-18) by in situ hybridization performed at 70°C using the m*Spry2* riboprobe. Transgene expression was detected in the nascent limb bud in 14/17 cases (data not shown). There was no cross-hybridization of the m*Spry2* probe with endogenous c*Spry2* RNA.

RESULTS

Isolation and evolutionary conservation of vertebrate Sprouty genes

cDNAs representing four mouse genes with sequence homology to *Drosophila* and human *spry* genes, and the chick orthologs of *Spry1* and *Spry2* were isolated. Sequence analysis indicated that all contained full coding regions except the m*Spry3* and c*Spry1* cDNA clones, which were partial cDNAs encoding only the C-terminal region of the proteins. The predicted mSPRY1, mSPRY2, and mSPRY4 proteins are similar in size (34, 34.6 and 32.6 kDa, respectively), and each contains an ~125 amino acid (aa) residue cysteine-rich C-terminal domain with at least 23 cysteines (Fig. 1). mSPRY3 contains a similar cysteine-rich domain (not shown). Throughout the C-terminal domain the four mouse proteins share 56-70% aa sequence identity. In the N-terminal domain mSPRY1 and mSPRY2 are more similar to one another (~37% aa identity) than mSPRY1 is to mSPRY4 (~25% aa identity) or mSPRY2 is to mSPRY4 (~25% aa identity). Comparison of the mouse, chick, and previously described human SPRY2 sequences demonstrates that the *Spry2* gene has been highly conserved during vertebrate evolution (m/h, 97% aa identity; m/c, 84% aa identity; h/c, 86% aa identity); most of the differences among species are in the N-terminal domain.

The similarity between the vertebrate and *Drosophila* sprouty (dSPRY) protein sequences (Fig. 1 and data not shown; Hacohen et al., 1998) is limited mostly to the cysteine-rich domain, with each vertebrate protein showing ~44-52% aa identity to dSPRY in this region. The cysteine residues are particularly highly conserved, with 18 or more of the 22 cysteines in the *Drosophila* protein present in each vertebrate gene. Outside the cysteine-rich domain there are two short regions that show similarity between dSPRY and all three mouse proteins (Fig. 1). There are also short stretches of sequence similarity between dSPRY and individual vertebrate Sprouty proteins, such as the region containing residues 345-356 in dSPRY and residues 80-91 in the human (Hacohen et al., 1998), mouse and chick SPRY2 proteins (data not shown). The many stretches of repeated or alternating amino acid residues found in dSPRY are not present in any of the vertebrate Sprouty family members. None of the vertebrate SPRY proteins contains a predicted signal peptide, whereas dSPRY has a signal sequence predicted by the GCG SPScan program near its N-terminal end. Thus, if the vertebrate SPRY proteins are secreted it may be by a signal peptide-independent mechanism (reviewed by Muesch et al., 1990; Friesel, et al., 1995).

The chromosomal map locations of m*Spry1*, m*Spry2*, and m*Spry4* genes were determined by standard backcross analysis. m*Spry1* mapped to proximal chromosome 3 and m*Spry2* to distal chromosome 14. Their human orthologs have been mapped to the syntenic regions of human chromosomes 4 and 13, respectively (Deloukas et al., 1998). m*Spry4* mapped to central chromosome 18. m*Spry3* was not mapped, but its human ortholog maps to the X chromosome (Rogner et al., 1996), suggesting that m*Spry3* might also be X-linked. None

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mSPRY1    MDSPS-QHGS HT-SLVVIQP PAVEGRQRLD -YDRDTQ-PA   36
mSPRY2    MEARA-QSGN GSQPLLQTAH DSGRQRGEPD PRDALTQ-QV   38
mSPRY4    MEPPVPQS-S F--PVNPSSV MVQPLLDSRA PHSRL-QHPL   36
dSPRY                                  (176)ALGQPPA

mSPRY1    TILSLDQIKA -I-RGSNEYT EGPSVARRPA PRTAPRPEKQ   74
mSPRY2    HVLSLDQIRA -I-RNTNEYT EGPTVVPRPG LKPAPRPSTQ   76
mSPRY4    TILPIDQMKT -S-HVENDYI DNPSLAPATG PKR-PRGGPP   73
dSPRY     SPVTLAQPRP ESERLTNEYV DTP (206)

mSPRY1    ERTHEIIPAN VNSSYEHRPA SHPGNARGSV LSRSTSTGSA 114
mSPRY2    HK-HERLHGL PEHRQPPRLQ PSQVHSSRAP LSRSISTVSS 115
mSPRY4    ----E-LAPT PA-RCDQDIT HHWISFSGRP -----SSVSS 102

mSPRY1    ASSGS---S- SSVSSEQ--G LLGRSPPTRP IPGHRSDRVI 148
mSPRY2    GSRSSTRTST SSSSSEQ--R LLGPSFSHGP AAADGI---I 150
mSPRY4    --------S- SSTSSDQ--R LLDHMAPPPV AEQASPRAV- 130
dSPRY          (284) T -SVGSDHTDG LL (295)

mSPRY1    RTQPKQLLV- E-DLKASL-K EDPTQHKFIC EQCGKCKCGE 185
mSPRY2    RVQPKSEL-K PGDVK-PLSK DDLGLHAYRC EDCGKCKCKE 188
mSPRY4    RLQPKVVHCK PLDLKGPTAP PELDKHFLLC EACGKCKCKE 170
dSPRY                          (380)C PRCGRCRCEQ 390

mSPRY1    CTAPRALPSC LACDRQ-CLC SAESMVEYGT CMCLVKGIFY 224
mSPRY2    CTYPRPLPSD WICDKQ-CLC SAQNVIDYGT CVCCVKGLFY 227
mSPRY4    CASPRTLPSC WVCN-QECLC SAQTLVNYGT CMCLVQGIFY 209
dSPRY     CQSPRPLPQT WVCNKT-CLC SAESVIDYAS CLCCAKALFY 429

mSPRY1    HCSNDDDGGS YSD-N----- --PCSCSQSH CCSRYLCMGA 256
mSPRY2    HCSNDDEDN- CAD-N----- --PCSCSQSH CCTRWSAMGV 258
mSPRY4    HCTNEDDEGS CAD-H----- --PCSCSGSN CCARWSFMGA 241
dSPRY     HCARDNDLD- CDDGNGTPCV DNPCSCGPYK RTQRWGWLGA 468

mSPRY1    LSLCLPCLLC YPPAKGCLKL CRGCYDWTHR PGCRCRNSNT 296
mSPRY2    MSLFLPCLWC YPPAKGCLKL CQGCYDRVNR PGCRCKNSNT 298
mSPRY4    LSVVLPCLLC YLPATGCVKL AQRGYDRLRR PGCRCKHTNS 281
dSPRY     LSIFLPCLWF YWPMRGCMKL CEKCYGRFAG RGCRC(503)

mSPRY1    VYCKLESCPS RAQG--KLS                        313
mSPRY2    VCCKVPTVPP RNFE--KPT                        315
mSPRY4    VICKAASGDT KTSRSDKPF                        300
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Fig. 1. Sequence comparison of three mouse and *Drosophila* Sprouty proteins. The deduced amino acid sequences of mouse SPRY1, SPRY2 and SPRY4 are aligned with portions of the *Drosophila* SPRY sequence (dSPRY residue numbers from Hacohen et al., 1998). Dashes represent gaps inserted to maximize alignment. Amino acids conserved in two or more Sprouty family members are highlighted in light gray. Cysteine residues are highlighted in dark gray. Asterisks indicate the residues conserved in all three mouse proteins.

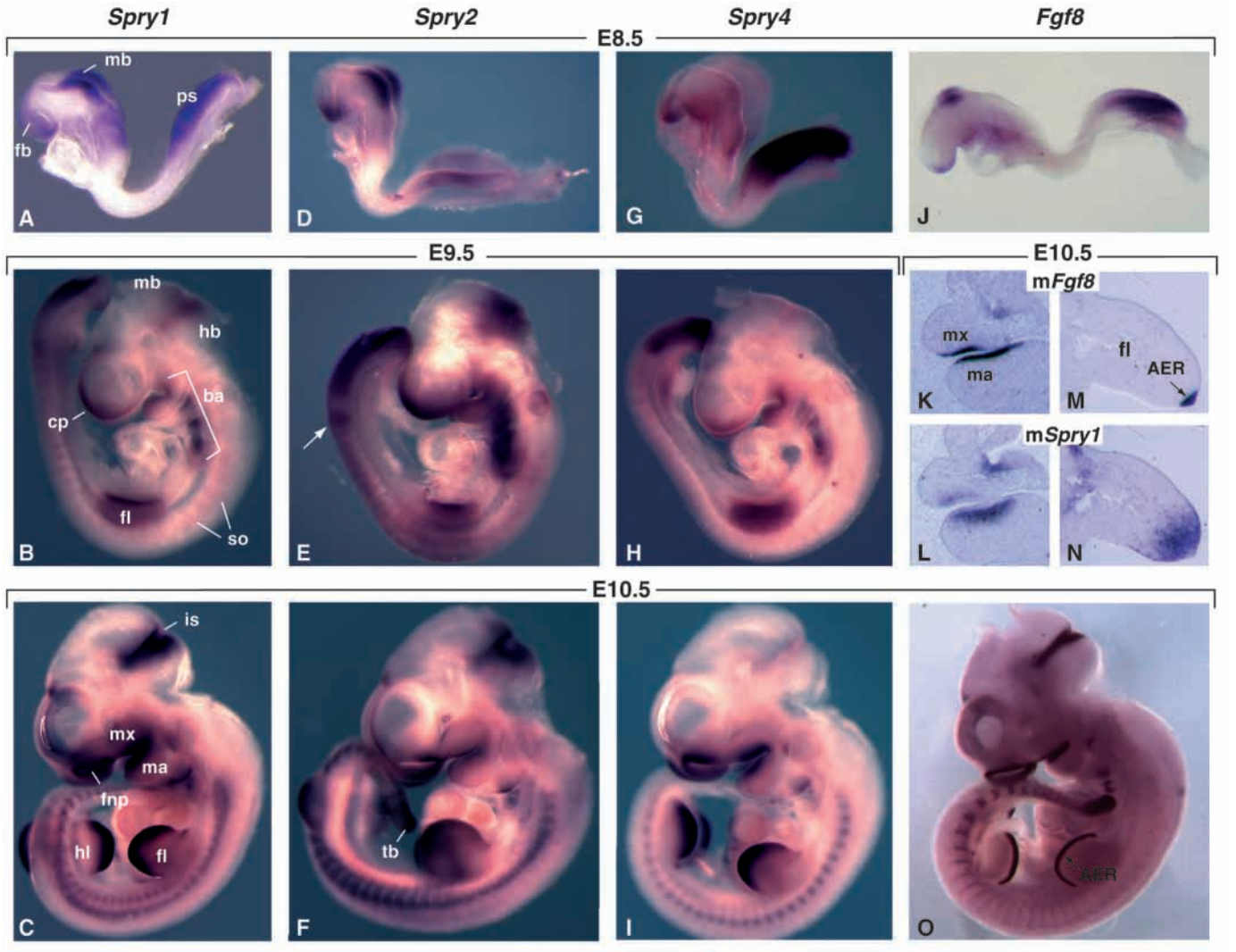

Fig. 2. Comparison of Sprouty gene expression patterns in the mouse embryo from E8.5 to E10.5. Analysis by whole-mount in situ hybridization for (A-C) m*Spry1*, (D-F) m*Spry2*, (G-I) m*Spry4*, and (J,O) *Fgf8* gene expression in mouse embryos at (A,D,G,J) E8.5, (B,E,H) E9.5 and (C,F,I,O) E10.5. (K-N) In situ hybridization of near adjacent paraffin sections of E10.5 mouse embryos. (K,L) Region containing the first branchial arch; (M,N) the forelimb. Near adjacent sections were hybridized with (K,M) m*Fgf8* or (L,N) m*Spry1*. The region of presomitic mesoderm in which the next somite will segment is marked by a distinct stripe of *Spry1* and *Spry2* but not *Spry4* expression (arrow in panel E and data not shown). AER, apical ectodermal ridge; ba, branchial arches; cp, commissural plate; fb, forebrain; fl, forelimb bud; fnp, frontonasal process; hb, hindbrain; hl, hindlimb bud; is, isthmic constriction; ma, mandibular arch; mx, maxillary arch; mb, midbrain; ps, primitive streak; so, somite; tb, tail bud.

of the mapped mouse Sprouty genes co-localize with a known mouse mutation.

Vertebrate Sprouty genes are expressed near FGF signaling centers in the embryo

Sprouty expression in mouse embryos and adult tissues was initially surveyed by northern blot analysis of poly(A)$^+$ RNAs. m*Spry1*, m*Spry2* and m*Spry4* RNAs were detected in the embryo and in many adult tissues including heart, brain, lung, kidney and skeletal muscle. For both m*Spry1* and m*Spry2*, there was a major ~2.5 kb transcript and a minor ~6.5 kb form, whereas for m*Spry 4* there was a major ~6 kb, a minor ~2 kb, and an additional ~7.2 kb transcript in skeletal muscle (data not shown). Although m*Spry3* expression was not detected in the embryo by northern blot (E7-E18) or whole-mount in situ hybridization (E7.0-E11.5) analysis, transcripts of ~6.5 kb and ~1 kb were detected in adult brain and testis, respectively (data not shown).

Whole-mount in situ hybridization analysis demonstrated that at stages when organogenesis is commencing in the mouse embryo (E8.5-E9.5), *Spry1*, *Spry2* and *Spry4* are expressed in similar, highly localized domains. At E8.5 expression of all three genes was detected in the primitive streak, in the rostral forebrain, and in cells lateral to the posterior hindbrain (Fig. 2A,D,G). At E9.5, expression continued to be detected in the rostral forebrain and primitive streak, and was also detected in the branchial arches and the forelimb bud (Fig. 2B,E,H, and data not shown). At E10.5, expression of the three genes was also detected in the somites, frontonasal processes, tailbud, and hindlimb bud (Fig. 2C,F,I, and data not shown). In addition to

these common expression domains, m*Spry1* and m*Spry2*, but not m*Spry4* RNAs were detected throughout the developing midbrain and in the anterior hindbrain at E8.5, and subsequently in a transverse domain that encompassed the prospective caudal midbrain, isthmus, and cerebellum (Fig. 2A-I), as well as in a distinct stripe that appeared to mark the region of presomitic mesoderm in which the next somite will segment (Fig. 2E, and data not shown). Thus, at least up to midgestation stages, *Spry1*, *Spry2* and *Spry4* are expressed in highly restricted patterns and appear to be co-regulated in most tissues. The chick *Spry1* and *Spry2* genes displayed similar expression patterns at comparable developmental stages (data not shown).

One important aspect of these expression patterns is that in both mouse and chick each of the Sprouty expression domains overlapped with or was immediately adjacent to the known expression domains of one or more FGF genes, with the possible exception of cells adjacent to the posterior hindbrain. This correlation was particularly evident when Sprouty and *Fgf8* gene expression patterns were compared (Fig. 2J-O). In some domains the Sprouty genes and *Fgf8* appeared to be co-expressed in the same cells, as for example, in the rostral forebrain and at the mid/hindbrain junction. However, in the latter region the Sprouty expression domains extended both rostral and caudal to the *Fgf8* expression domain (compare Fig. 2C,F with O). In other regions Sprouty gene expression was detected exclusively in cells adjacent to cells expressing *Fgf8*. For example, m*Fgf8* RNA is detected in the ectoderm of the mandibular and maxillary arches (Fig. 2K) and m*Spry1* RNA in the subadjacent mesenchyme (Fig. 2L). In the limb bud, m*Fgf8* RNA is detected in the AER (Fig. 2M) and m*Spry1* RNA in the underlying mesenchyme (Fig. 2N). In these tissues, there appeared to be a gradient of Sprouty gene expression, with the highest levels in cells closest to the FGF signaling center. These results suggest that FGFs act as inducers of Sprouty gene expression.

FGF signaling induces Sprouty gene expression

To determine if FGF signaling can induce Sprouty gene expression we examined the effects of adding or removing FGF signals in various developmental settings on the expression of the Sprouty genes. We first tested whether FGF signaling was sufficient to induce ectopic Sprouty gene expression in two tissues in the chick embryo known to be responsive to addition of FGFs (Cohn et al., 1995; Crossley et al., 1996a), but in which *Spry1* and *Spry2* are not normally expressed. When a heparin bead soaked in recombinant FGF4 (FGF4-bead) was implanted in the prospective caudal diencephalon at ~stage 11, *Spry2* RNA was detected in the neuroepithelium surrounding the bead in most (9/11) cases (Fig. 3A), whereas no such induction occurred with control beads soaked in PBS (PBS-beads) (n=3; data not shown). Similarly, both *Spry1* (n=5) and *Spry2* (n=8) expression were induced in cells near an FGF4-bead implanted in the lateral plate mesoderm (LPM) of the chick interlimb region at stage 14, (Fig. 3B, and data not shown). FGF2-beads (n=3) and FGF8-beads (n=3) also induced Sprouty gene expression, whereas PBS-beads had no effect (Fig. 3C, and data not shown). A time course analysis showed that *Spry2* expression could be induced within one hour after implantation of an FGF4-bead (data not shown). Thus FGF signaling can rapidly induce Sprouty gene expression, suggesting it is a primary response to the signal.

To determine the relationship between FGF signaling and endogenous Sprouty gene expression, we first examined Sprouty expression in explant cultures of the mouse mandibular arch (Neubüser et al., 1997). All three Sprouty genes are normally expressed in mandibular arch mesenchyme,

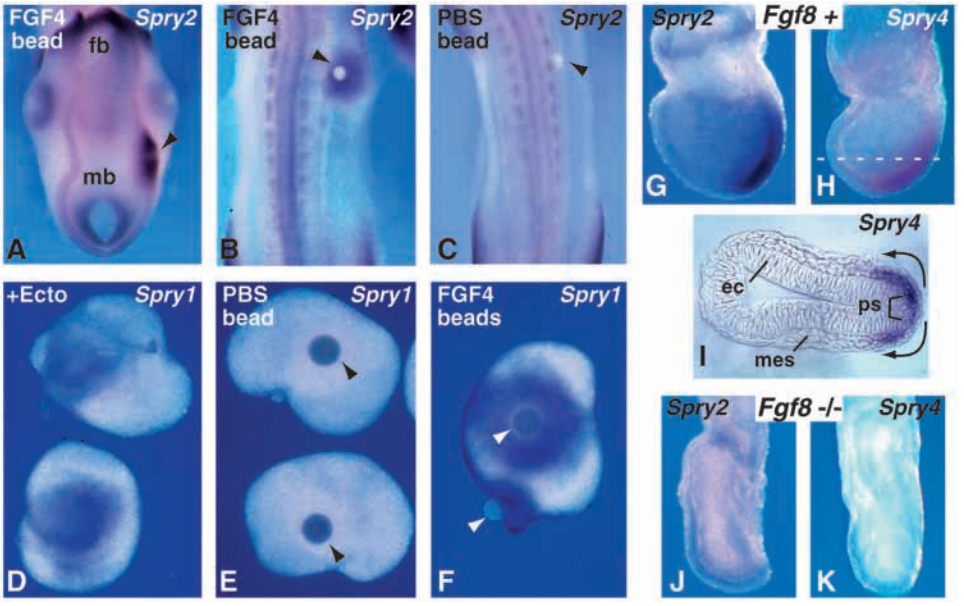

Fig. 3. FGF signaling induces and is required for Sprouty gene expression. (A-C) Induction of ectopic Sprouty gene expression following implantation of FGF4-beads in the brain or interlimb region of the chick embryo in ovo. (A) Dorsal view of the brain assayed for c*Spry2* expression 24 hours after bead implantation into the caudal diencephalon at approx. stage 11. (B,C) Dorsal views of embryos assayed for *Spry2* expression 24 hours after implantation of (B) an FGF4-bead or (C) a control PBS-bead into the flank of stage 14-15 embryos. (D-F) Dependence on FGF signaling of *Spry1* expression in the mandibular arch. Assays by whole-mount in situ hybridization for *Spry1* RNA in explants of the mouse mandibular arch cultured for 24 hours after the surface ectoderm was removed and (D) immediately reattached to the mesenchyme; (E) a control PBS-bead was implanted; (F) FGF4-beads were implanted. (G-K) Absence of Sprouty gene expression in the primitive streak (ps) of $Fgf8^{-/-}$ mouse embryos. Assays by whole-mount in situ hybridization for (G,J) *Spry2* and (H,K) *Spry4* expression in (G,H) non-mutant (*Fgf8*+) or (J,K) mutant ($Fgf8^{-/-}$) E7.5 embryos. (I) Transverse section of the normal embryo shown in H (dotted line shows the approximate plane of section). *Spry4* RNA is detected in the ps region, with highest levels in nascent mesoderm cells. Sprouty gene expression is downregulated as mesodermal cells (mes) migrate away from ps (arrows indicate the direction of cell migration), and is not detected in the embryonic ectoderm (ec). Arrowheads point to the beads. Abbreviations as in Fig. 2.

in the region immediately underlying ectoderm that expresses *Fgf8* (see Fig. 2K) and *Fgf9* (Kettunen and Thesleff, 1998). When the mandibular ectoderm was left in place (*n*=5) or removed and then immediately replaced (*n*=10), *Spry1* RNA was detected in the underlying mesoderm (*n*=13/15; Fig. 3D). In contrast, when the mandibular ectoderm was removed and a PBS-bead was implanted, *Spry1* RNA was not detected (*n*=9; Fig. 3E), indicating that signals from the overlying ectoderm are required to maintain *Spry1* expression. However, when an FGF4-bead was implanted in the isolated mesenchyme, *Spry1* RNA was detected in all cases (*n*=10), but only in the region surrounding the FGF4-bead (Fig. 3F). These data indicate that FGF signaling is sufficient to maintain Sprouty gene expression in the mandibular arch. Since *Fgf4* is not expressed in the mandibular arch ectoderm (Kettunen and Thesleff, 1998), it is likely that *Fgf8* and/or *Fgf9* expressed there provide the endogenous signals that regulate Sprouty gene expression.

We next examined Sprouty gene expression in *Fgf8* null mutant mouse embryos at E7.5. At this stage *Fgf8* RNA is detected in the primitive streak (Crossley and Martin, 1995), where ectodermal cells undergo a transition from an epithelial to mesenchymal morphology and generate the mesodermal and endodermal lineages. *Spry2* and *Spry4* but not *Spry1* RNAs are also detected in the primitive streak at this stage (Fig. 3G,H, and data not shown), with the highest levels localized in the nascent mesoderm (Fig. 3I). *Spry2* and *Spry4* expression appear to be rapidly downregulated as the cells migrate away from the streak (Fig. 3I, and data not shown). *Fgf8*$^{-/-}$ embryos fail to gastrulate normally, but the primitive streak forms and nascent mesoderm cells are apparently present (Sun et al., 1999). However, neither *Spry2* (*n*=5) nor *Spry4* (*n*=5) RNA was detected in the nascent mutant mesoderm (Fig. 3J,K). Together the data from these experiments demonstrate that expression of all three Sprouty genes is dependent on FGF signaling.

Ectopic Sprouty gene expression inhibits limb bud outgrowth and causes chondrodysplasia

To explore vertebrate Sprouty gene function, we expressed Sprouty genes throughout the chick limb and analyzed the effects at various stages of limb development. In the normal limb bud, all three Sprouty genes are expressed in the distal mesenchyme underlying the AER, which expresses *Fgf8* (Fig. 2). The AER also expresses *Fgf4* (Niswander and Martin, 1992), *Fgf9* and *Fgf17* (M. Lewandoski and G.R.M., unpublished observations). If Sprouty functions as an antagonist of FGF signaling in vertebrates as it does in *Drosophila,* then one might expect excess Sprouty gene expression to interfere with FGF-mediated outgrowth and patterning of the limb.

The prospective forelimb territory was infected at stages 9-10 (E1.5) with a replication-competent avian retrovirus containing an m*Spry2* cDNA, and the infected embryos were allowed to develop for up to 4.5 days after infection (up to stage 28; E6). In 56 embryos (52%), there was a modest reduction and in 8 embryos (7%) a dramatic reduction in limb bud size compared to the contralateral uninjected wing (Fig. 4A-D). In the remaining 45 embryos there was no obvious effect on wing bud size (data not shown). Similar results were obtained with m*Spry4* virus: 17 of the 26 infected limb buds (65%) were modestly reduced in size (data not shown). No effect was observed in the uninfected limb, or in limbs infected with a control retrovirus containing a GFP cDNA (GFP virus; *n*=20). The affected wing buds were assayed for expression in the distal mesenchyme of genes that are dependent on FGF signals from the AER, such as *Shh, Fgf10, Msx1, Bmp2* or *Bmp4* (reviewed by Martin, 1998). Expression of each of these genes was detected in all the infected limb buds examined (Fig. 4B, and data not shown). In most affected wing buds, AER morphology and gene expression patterns (*Fgf4, Fgf8, Bmp2* and *Bmp4)* appeared normal (data not shown), although in some cases *Fgf8* expression was patchy (Fig. 4C). This suggests that Sprouty misexpression can interfere with a signal from the mesenchyme that maintains *Fgf8* expression, possibly FGF10 (Min et al., 1998; Sekine et al., 1999).

The effects of excess Sprouty expression on the patterning processes that occur during the bud stages of development were determined by examining skeletal preparations of embryos incubated for approx. 5-12 days after retroviral infection (to stages 30-39; E6.5-E13.5). In 44 of 60 (73%) embryos infected with the *Spry2* virus, but in none of the embryos infected with the control GFP virus (*n*=9), the injected wing was much smaller than the uninjected one. In 2 of the 44 cases (5%), the stunted wing lacked the normal complement of skeletal elements: the only skeletal element present was a small humerus (Fig. 4E). The absence of skeletal elements distal to the humerus is similar to what is observed when the AER is removed at early stages of limb bud outgrowth. As discussed below, these data support the conclusion that excess Sprouty expression in the limb can antagonize FGF signaling from the AER.

The remaining 42 (95%) affected wings, although not lacking any skeletal elements, had a striking phenotype: the skeletal elements were considerably shorter and usually thicker than those in the uninjected limb (Fig. 4F). In 31 cases (70%) all the elements were thus affected, and in 11 cases (25%) only some were affected, presumably because the limb was only partially infected (data not shown). To determine whether the decreased size of the skeletal elements could be attributed to reduction in the size of the mesenchymal condensations from which they developed, we examined the expression of *Sox9*, an early marker of such condensations (Wright et al., 1995), in stage 23-28 infected embryos. In most cases (*n*=15) the condensations in the infected wing were indistinguishable or only modestly reduced in size compared with those in the uninjected wing (Fig. 4D). This result suggested that the reduction in skeletal element size was due to abnormalities in the orderly progression from proliferating to hypertrophic chondrocytes that is essential for normal endochondral bone elongation.

Hypertrophic chondrocytes are normally localized towards the center of a skeletal element and are readily detected by their characteristic morphology (Fig. 4G,I). Analysis of serial sections showed that affected wings at stages 34-39 contained no cells with the distinctive histological characteristics of hypertrophic chondrocytes (Fig. 4H,J, and data not shown). Consistent with this observation, *ColX* expression, a marker for hypertrophic chondrocytes (Vortkamp et al., 1996), was not detected in the affected skeletal elements (data not shown). Furthermore, no evidence of bone formation was detected after staining with Alizarin red, even in embryos incubated to stage 39 (Fig. 4F), approximately 6.5 days after ossification begins

in the normal chick limb. This is consistent with studies indicating that differentiation of hypertrophic chondrocytes is required for invasion of cartilage by osteogenic cells (Karsenty, 1998). To determine whether chondrocytes progressed to the prehypertrophic stage, we assayed for *Ihh* expression (Vortkamp et al., 1996). Although *Ihh* was readily detected in the skeletal elements of the contralateral uninfected limb (Fig. 4K), *Ihh* RNA was not detected in the affected skeletal elements of the infected limb (Fig. 4L). This suggests that overexpression of *Spry2* prevents differentiation of proliferating chondrocytes. Consistent with this conclusion, proliferating cells, as detected by BrdU labeling, which are normally restricted to the ends (Fig. 4M), were found scattered throughout the affected skeletal elements (Fig. 4N). Thus, excess expression of Sprouty during chick skeletal development causes a severe chondrodysplasia in which cartilage cell differentiation is inhibited.

DISCUSSION

In *Drosophila*, FGF signaling induces expression of its own antagonist, *sprouty*. By isolating mouse Sprouty genes and their chick orthologs and studying their expression in normal embryos and various experimental settings we have demonstrated that this regulatory relationship has been conserved. In many embryonic tissues, Sprouty genes are expressed in intimate association with FGF signaling centers. Evidence that Sprouty expression is dependent on FGF signaling was obtained from studies showing that *Spry1* expression is not maintained in branchial arch mesenchyme deprived of FGF-expressing ectoderm unless an FGF-bead is applied, and that *Spry2* and *Spry4* are not detected in the primitive streak of *Fgf8*$^{-/-}$ embryos. Moreover, *Spry1* and *Spry2* are ectopically expressed in the chick brain and interlimb region in response to an FGF-bead. In *Drosophila*, FGF induction of *sprouty* expression appears to be a primary response mediated by a MAP kinase cascade (Hacohen et al., 1998). In vertebrates, FGF induction of Sprouty gene expression is very rapid, suggesting it is also a primary response to the signal. Evidence that Sprouty genes antagonize FGF signaling was obtained from studies of excess Sprouty expression in the developing limb. We observed a reduction in limb bud size and in some cases limb truncation consistent with inhibition of the FGF signal required for limb bud outgrowth and patterning. In addition, overexpression of Sprouty inhibited chondrocyte terminal differentiation. This caused a chondrodysplasia similar to that observed in patients with mutations in FGFR3, raising the possibility that Sprouty plays a role in some human chondrodysplasias.

Regulation of Sprouty gene expression by FGF signaling

Our data show that from gastrulation through mid-gestation stages of development, *Spry1*, *Spry2* and *Spry4* have remarkably similar and highly specific expression domains, suggesting that these three genes may be coordinately regulated. We have observed a striking correlation between the expression domains of the Sprouty genes and *Fgf8* during this period of development, suggesting that FGF8 may regulate Sprouty gene expression. The absence of Sprouty gene expression in the primitive streak of *Fgf8*$^{-/-}$ embryos supports this hypothesis. However, other FGF family members are also expressed in each domain and could participate in the regulation of Sprouty genes. For example, *Fgf4* expression, which is detected in the primitive streak of normal but not *Fgf8*$^{-/-}$ embryos (Niswander and Martin, 1992; Sun et al., 1999), might be required for Sprouty gene expression. On the other hand, since *Fgf8*$^{-/-}$ embryos express *Fgf5* (Sun et al., 1999), it appears that not all FGFs induce Sprouty gene expression. At later stages of development Sprouty gene expression is detected in specific cell types in virtually every organ, including the eye, lung, gut, brain, kidney and bone (data not shown; de Maximy et al., 1999), and the correlation between Sprouty and *Fgf8* gene expression does not always hold.

Although there is extensive overlap in the expression domains of the individual Sprouty genes up to mid-embryogenesis, there are significant differences among them. For example, *Spry2* and *Spry4* are expressed in the primitive streak whereas *Spry1* is not, and *Spry 1* and *Spry2* are expressed in the midbrain and anterior hindbrain region whereas *Spry4* is not. Such differences might be due to differences among Sprouty genes in their sensitivity to FGF signaling or perhaps differences in other factors required for individual Sprouty gene expression.

The functional significance of multiple Sprouty gene expression in a given domain remains to be determined. One possibility is that different Sprouty genes have different effects on FGF signaling pathways or possibly other RTK signaling pathways active in the domain. Alternatively, the effects of different Sprouty proteins may be similar, perhaps serving to restrict the range of FGF signaling activity as in the *Drosophila* tracheal system, and the requisite amount of Sprouty activity in a domain may be achieved by co-expression of several different Sprouty genes. Excess m*Spry2* or m*Spry4* in the developing chick limb bud caused similar phenotypes, indicating that these two genes have similar effects, at least when overexpressed.

Sprouty can antagonize FGF function in the limb bud

We assessed the function of Sprouty gene products in the chick limb bud at stages 18-28, when its development is dependent on FGFs produced in the AER, by using a retroviral vector to express *Spry2* or *Spry4* genes and examining the effects of Sprouty overexpression on limb bud outgrowth, marker gene expression, and skeletal element specification. In the infected limb buds, we observed a modest to severe reduction in bud outgrowth, but the continued expression of genes known to be dependent on FGF signaling. These results are different from the effects observed when all FGF signaling is eliminated by removing the AER. However, they are similar to what has been observed when the *Fgf8* gene is specifically inactivated in the AER of the mouse embryo limb bud (M. Lewandoski and G. R. M., unpublished observations). On the basis of these similarities, we suggest that the Sprouty expression obtained by retroviral infection of chick limb buds was in most cases sufficient to antagonize but not to eliminate FGF signaling. However, Sprouty expression levels may have been close to the threshold needed to eliminate FGF signaling, since a small proportion of the limbs ultimately displayed a skeletal phenotype similar to that obtained when the AER is removed

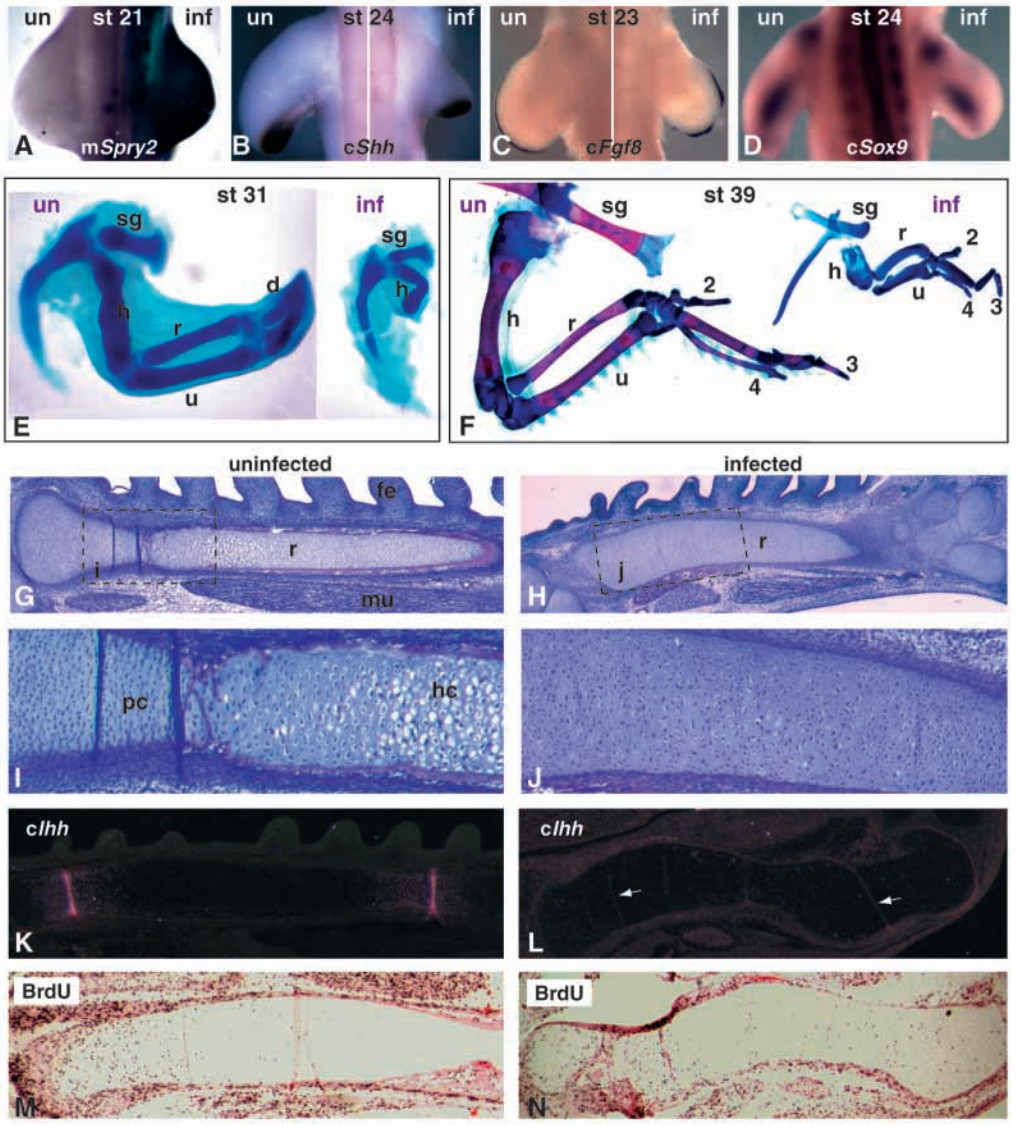

Fig. 4. Effects of Sprouty overexpression on limb development. The prospective right wing territory of stage 9-10 chick embryos was infected with an m*Spry2* retrovirus. (A-D) Dorsal views of infected embryos assayed by whole-mount in situ hybridization at the stages indicated using the probes indicated. Note the reduced size of the infected (inf) as compared to the contralateral, uninfected (un) limb bud and, in A, the widespread expression of the m*Spry2* transgene in the infected limb bud. (E) Skeletal preparation of the wings of a stage 31 infected embryo stained with Alcian blue. All elements distal to the humerus are absent in the infected wing, whereas the contralateral uninfected wing is normal. (F) Skeletal preparations of the wings of stage 39 infected embryos stained with Alizarin red and Alcian blue. Note that the infected wing contains all the skeletal elements present in the normal wing including three digits (d; by convention numbered 2, 3, and 4), but fails to stain with Alizarin red, indicating the absence of bone formation. (G-N) Horizontal sections through the uninfected or infected radius, as indicated, of a stage 35 embryo. (G-J) Sections stained with H&E. Boxes indicate areas shown at higher power in I and J. Note the absence of hypertrophic chondrocytes (hc) in the radius of the infected wing. (K,L) Sections hybridized with a probe for c*Ihh*. *Ihh* expression was detected in the uninfected but not in the infected radius. The intensely labeled vertical lines within the *Ihh* expression domains in the uninfected radius are folds in the tissue. Similar folds in the infected radius, indicated by arrows, are not labeled. (M,N) Sections from an embryo injected with BrdU. Additional abbreviations: fe, feather bud; h, humerus; mu, muscle; pc, proliferating chondrocytes; r, radius; sg, shoulder girdle; u, ulna.

at stages 18-19: development of the humerus but not more distal skeletal elements (Saunders, 1948; Summerbell, 1974). These effects might be due to interference with either FGFR1 or FGFR2 signaling pathways, since both of these receptor genes are expressed in limb bud mesenchyme (Peters et al., 1992; Xu et al., 1998).

In the normal limb bud, Sprouty genes are expressed in the mesenchyme underlying the AER, consistent with their induction by FGF signaling from the AER. This region contains the “progress zone,” in which the cell division responsible for limb bud outgrowth and the specification of limb skeletal elements occurs (Summerbell et al., 1973). If Sprouty proteins inhibit FGF signaling from the AER, as our overexpression studies suggest, then Sprouty genes may serve as FGF feedback inhibitors during normal limb bud development, modulating the rate of cell proliferation or perhaps limiting the range of FGF signaling from the AER to the progress zone, much as dSPRY limits the range of FGF signaling in *Drosophila* tracheal development. This model predicts that in Sprouty loss-of-function mutants limb bud outgrowth and skeletal element patterning will be perturbed.

The effects of Sprouty overexpression were first observed during the bud stages of limb development (stages 18-28). We did not observe effects on limb induction and initiation of limb bud formation, processes that are also thought to depend on FGF signaling (reviewed by Martin, 1998) but which occur at stages 13-17. This was not surprising because the recombinant virus was introduced at stages 9-10, and retroviral gene expression does not become robust until 18-24 hours after infection (stage 17-18) (Morgan and Fekete, 1996).

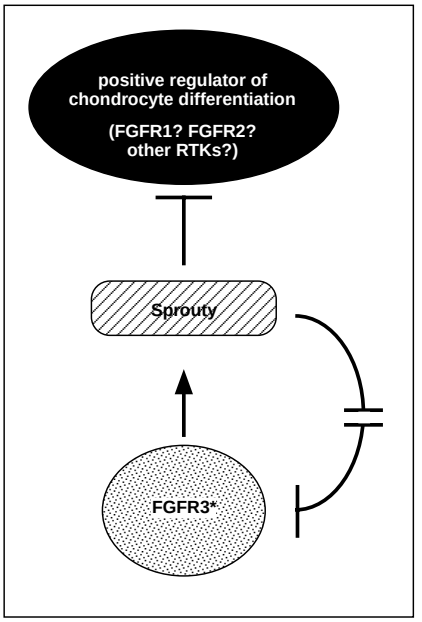

Fig. 5. A model for SPROUTY function in chondrodysplasias caused by activating mutations in FGFR3. The mutant FGFR3 protein (FGFR3*) induces expression of one or more Sprouty genes, which in turn inhibits a positive regulator(s) of bone elongation, but not FGFR3*, which functions as a negative regulator of bone elongation.

Sprouty overexpression causes chondrodysplasia

Analysis of limbs overexpressing Sprouty at stages when skeletal element elongation and ossification normally occur (stages 30-39; E6.5-E13.5), revealed that approximately two-thirds of the infected limbs display a striking chondrodysplasia. All skeletal elements are present but each is dramatically shorter than normal. This is apparently due to a block in the differentiation of proliferating chondrocytes, which results in the lack of chondrocyte hypertrophy and subsequent bone formation.

FGF signaling is known to be a negative regulator of endochondral bone formation. In *Fgfr3*$^{-/-}$ mice, the hypertrophic zone is enlarged and there is excessive bone elongation (Colvin et al., 1996; Deng et al., 1996). If Sprouty always functions as an antagonist of all FGF signaling, one would predict that Sprouty overexpression in skeletal elements would mimic the effects of loss of FGFR3 signaling. However, the effects of Sprouty overexpression in the chick limb mimicked or were more severe than those caused by excess FGF signaling (Coffin et al., 1995; Vogel et al., 1996). The gross morphologic and histologic features of the affected limbs were similar to those seen in several human chondrodysplasias caused by mutations in *Fgfr3* that result in ligand-independent signaling activity by the receptor (Horton and Hecht, 1993; Webster and Donoghue, 1997; Burke et al., 1998). Fetuses heterozygous for the most strongly activating mutations are afflicted with type I Thanatophoric Dysplasia (TD), the most common form of lethal congenital dwarfism, and have short, thick, excessively curved femurs, and a paucity of hypertrophic chondrocytes. Heterozygosity for less strongly activating mutations in *Fgfr3* are associated with the most common form of congenital dwarfism, Achondroplasia (Ach). Fetuses homozygous for such mutations have a phenotype that closely resembles type I TD. Recent studies show that the skeletal elements in transgenic mice overexpressing an *Fgfr3* mutant allele, similar to the one that causes Ach, display a decrease in the hypertrophic and proliferative zones, and a modest reduction in bone length (Naski et al., 1998). One possible explanation for our results is that in developing bone, and perhaps other developmental settings, Sprouty might function as an enhancer rather than an antagonist of FGFR3 signaling.

Alternatively, Sprouty overexpression may have no effect on signaling via FGFR3, but instead may antagonize other FGFR signaling pathways that function as positive regulators of endochondral bone formation. Presently, there is no genetic evidence that FGF signaling positively regulates chondrocyte differentiation, but *Fgfr1* is expressed in osteoblasts and hypertrophic chondrocytes, and *Fgfr2* is expressed in the perichondrium (Peters et al., 1992). Their potential roles in skeletal development are not known because severe loss-of-function *Fgfr1* and *Fgfr2* mutations cause lethality before the bones form (Deng et al., 1995; Yamaguchi et al., 1995; Arman et al., 1998; Xu et al., 1998). FGFR4 is an unlikely candidate because loss-of-function studies indicate that it is not required for bone development (Weinstein et al., 1998). It is also possible that the chondrodysplasia that we observed is due to Sprouty-mediated inhibition of EGFR or some other RTK signaling pathway (Bergmann et al., 1998; Casci et al., 1999; Kramer et al., 1999) that positively regulates endochondral bone formation. One prediction of this model, consistent with our observation that FGF signaling induces Sprouty gene expression, is that the activating mutations in *Fgfr3* that cause human chondrodysplasias result in excess expression of one or more Sprouty genes. The resulting high levels of Sprouty protein would then inhibit an FGF signaling pathway (other than FGFR3) or some other RTK pathway required for normal endochondral ossification (Fig. 5). Tests of this hypothesis will require analysis of skeletal tissue from individuals with FGFR3-mediated chondrodysplasias, as well as a better understanding of the expression and functions of Sprouty and FGF receptor genes in normal skeletal development.

We are extremely grateful to Drs Cliff Tabin and Connie Cepko and members of their laboratory groups for instruction in methods of retrovirus construction and infection. We also thank Cliff Tabin for his generosity, encouragement and advice. We are also grateful to A. Gannon, L. Prentice, and M. Embry for excellent technical assistance, and Drs T. Vu, A. Erlebacher, and D. Leonardo for technical advice. We thank Dr Sidney Strickland, Stephanie Toering, and our laboratory colleagues for helpful comments and critical reading of the manuscript. Contributors to this work were supported by: a KO8 Award from the NCI (G. M.); training grant CA09302, awarded by the NCI (L. J.); a predoctoral fellowship from the NSF (C. C.); and postdoctoral fellowships from the HFSP (A. N.) and the American Cancer Society (X. S.). M. A. K. is an investigator of the Howard Hughes Medical Institute. This work was supported by the HHMI (M. A. K.) and NIH grant R01 CA78711 (to G. R. M.).

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