Cloning and characterization of a secreted frizzled-related protein that is expressed by the retinal pigment epithelium

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INTRODUCTION

The Wnt/frizzled cell signaling pathway has been implicated in the determination of polarity in a number of systems, including the Drosophila retina. The vertebrate retina develops from an undifferentiated neuroepithelium into an organized and laminated structure that demonstrates a high degree of polarity at both the tissue and cellular levels. In the process of searching for molecules that are preferentially expressed by the vertebrate retinal pigment epithelium (RPE), we identified secreted frizzled-related protein 5 (SFRP5), a member of the SFRP family that appears to act by modulating Wnt signal transduction. SFRP5 is highly expressed by RPE cells, and is also expressed in the pancreas. Within the retina, the related molecule SFRP2 is expressed specifically by cells of the inner nuclear layer. Thus, photoreceptors are likely to be bathed by two opposing gradients of SFRP molecules. Consistent with SFRP5’s postulated role in modulating Wnt signaling in the retina, it inhibits the ability of Xwnt-8 mRNA to induce axis duplication in Xenopus embryos. The human SFRP5 gene consists of three coding exons and it maps to chromosome 10q24.1; human SFRP2 maps to 4q31.3. Based on the biology and complementary expression patterns of SFRP2 and SFRP5, we suggest that they may be involved in determining the polarity of photoreceptor, and perhaps other, cells in the retina.

INTRODUCTION

Cell–cell communication, both during development and in the adult organism, is often mediated by interaction between secreted ligands and transmembrane receptor molecules. One important family of evolutionarily conserved signaling molecules, the Wnt family, consists of a group of cysteine-rich glycosylated ligands (1–3). Wnts have been implicated as being important in a variety of cellular processes, including embryonic induction, cell fate determination, control of cell polarity and malignant transformation. At least 16 different mammalian Wnt molecules have been identified. In general, they contain 350–400 amino acid residues, show at least 18% sequence identity to other family members and demonstrate a conserved pattern of 23–24 cysteine residues (1–3).

Recent evidence suggests that the transmembrane receptors for some, and perhaps all, Wnts belong to the frizzled family (4–6). Drosophila Schneider cells, which normally are unresponsive to Wingless (Wg), the Drosophila Wnt prototype, acquire the ability to bind Wg at their cell surface and become responsive to Wg when transfected with the Drosophila frizzled gene Dfz2 (4). Xenopus embryos, which normally do not undergo axis duplication in response to Wnt-5A, demonstrate axis duplication and develop an ectopic Spemann organizer when human frizzled-5 mRNA is co-injected with the Wnt-5A mRNA (6). In addition, overexpression of rat frizzled-1 in Xenopus embryos leads to increased surface immunostaining for Xwnt-8 (5).

The first member of the frizzled family was identified initially as a Drosophila tissue polarity-determining gene (7,8). Homologs have since been identified in a wide variety of species, including birds, fish, nematodes, sea urchins and mammals (9–11). In mammals, there are at least nine family members. All frizzled proteins contain an extracellular cysteine-rich domain (CRD) and seven putative membrane-spanning domains. The CRD contains 10 conserved cysteine residues. It is both necessary and sufficient for Wg binding, and thus it appears to constitute the ligand-binding domain (4).

The large number of Wnt and frizzled molecules expressed by individual organisms suggests the existence of a complex signaling network that is not yet well understood. The simplest hypothesis to account for the diversity of ligands and receptors is
that there is specificity in terms of which ligand interacts with which receptor, and specificity in terms of what signal is transduced. Consistent with this hypothesis, some of the Wnt and frizzled molecules show distinct expression patterns. For example, Wnt-3 is expressed in the thalamus, Purkinje cells in the cerebellum, thepons and shaft hair root in skin, while Wnt-5B is expressed in heart, liver, brain, lung, testis and placenta (12). Similarly, murine frizzled-3 (Mfz:3) is expressed most highly in the brain, whereas Mfz:5 is most strongly expressed in the eye, kidney and lung (10). Functionally, there are also differences. Class I Wnts (which includes Wnt-1, -3A, -8 and -8B) are capable of inducing axis duplication in Xenopus embryos and transformation of mammalian cell cultures, whereas class II Wnts (Wnt-4, -5A and -11) do not demonstrate these activities (13,14).

An additional layer of complexity in Wnt/frizzled signaling is suggested by the recent identification of a novel family of genes, the secreted frizzled-related proteins (SFRPs) (15–23). The SFRP genes encode secreted proteins that contain an N-terminal signal peptide, a frizzled-related CRD and a C-terminal hydrophilic region with some homology to the netrins, but lack evidence of any transmembrane domains. To date, five different mammalian SFRP genes have been identified. Because a number of groups identified many of the SFRPs almost simultaneously, SFRP nomenclature is confused by the use of several different names for the same genes (Table 1).

### Table 1. Names of the various secreted frizzled-related proteins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Proteins</th>
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<tbody>
<tr>
<td>SFRP1</td>
<td>sFRP-1 (15)</td>
</tr>
<tr>
<td>SFRP2</td>
<td>sFRP-2 (15)</td>
</tr>
<tr>
<td>SFRP3</td>
<td>sFRP-3 (15)</td>
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<tr>
<td>SFRP4</td>
<td>sFRP-4 (15)</td>
</tr>
<tr>
<td>SFRP5 (this study)</td>
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</table>

References are shown in parentheses.

*Human gene symbol approved by the human gene nomenclature committee.

The SFRPs appear to act as soluble modulators of Wnt signaling, presumably by competing with membrane frizzled receptors for the binding of secreted Wnt ligands. Although direct solution binding assays to assess SFRP–Wnt interactions have not yet been reported, membrane-tethered SFRPs have been shown to bind soluble Wg (15), and secreted SFRP3/Frzb can bind membrane-immobilized Wnt-1 (16). In addition, SFRPs and Wnts can be co-immunoprecipitated (17,24). mRNA for SFRP3/Frzb-1 interferes with muscle and trunk formation in Xenopus microinjection experiments. In co-injection studies, SFRP3/Frzb-1 mRNA blocks the ability of both Wnt-1 and Xwnt-8 to induce secondary axis formation (16,17). In these experiments, SFRP3/Frzb-1 shows some degree of specificity in that it does not inhibit signaling by Wnts-3A, -5A and -11. In human embryonic kidney cells, SFRP3/Frzb-1 reduces Wnt-1-induced cytoplasmic accumulation of β-catenin, which is thought to be important in Wnt signaling (24).

The neurosensory retina is responsible for visual signal transduction and initial image processing. The retinal pigment epithelium (RPE), which shares a common embryonic origin with the neurosensory retina, is important in maintaining the health and function of retinal photoreceptors. In the process of cloning and characterizing genes which are differentially expressed in the RPE, we identified SFRP5 which at the time was a novel member of the SFRP family. Here, we localize the expression of SFRP2 within the retina, provide novel information about the expression pattern of SFRP5, describe the genomic structure of mouse and human SFRP5, identify the human chromosomal location of SFRP2 and SFRP5, and demonstrate that SFRP5 can inhibit the axis-duplicating activity of Xwnt-8 in Xenopus embryos.

### RESULTS

#### Identification of SFRPs that are expressed preferentially in the retina and RPE

Partial sequence analysis of >1000 clones from a subtracted bovine RPE cDNA library that is enriched for genes that are preferentially expressed in the RPE and/or retina (25; J.T. Chang et al., in preparation) identified several clones that showed statistically significant homology to the Drosophila frizzled gene. Alignment and assembly of these clones suggested that they represented transcripts from two different genes. One set showed high homology to a human expressed sequence tag (EST), and was later identified as the mouse homolog of human SFRP2 (15). The other sequence at the time did not appear to correspond to any known gene. A full-length cDNA clone was isolated by screening a bovine RPE cDNA library. Its sequence indicated that it was a member of the SFRP family. Based on the nomenclature of Rattner et al. (15), and the gene symbols approved by the human gene nomenclature committee, we named this sequence SFRP5. [Subsequent to these experiments, the independent cloning of SFRP5 from a pancreas cDNA library was reported; it is referred to in that work as SFRP-3 (21)]. The sequences of murine, bovine and human SFRP5 predict open reading frames of 314, 315 and 317 amino acid residues, respectively, with a putative N-terminal signal sequence followed by a region homologous to the frizzled CRD (Fig. 1). The greatest sequence variability is in the putative signal sequence. Comparison of the predicted amino acid sequences of SFRP5 with the other known mammalian SFRPs is shown in Figure 1. (The full-length sequence of murine SFRP4, which had not been published previously, was obtained by sequencing a murine EST clone.) Although the murine, bovine and human SFRP5s are highly conserved (human–bovine, human–murine and bovine–murine comparisons indicate 98, 95 and 94% amino acid identity, respectively), except for the cysteine residues, there is only limited conservation that is maintained throughout the SFRP family. The closest homolog of SFRP5, SFRP1, shows 51% amino acid identity. SFRP2, SFRP3 and SFRP4, however, show only 36, 19 and 17% identity with SFRP5, respectively.

#### SFRP2 is expressed in the inner nuclear layer of the retina; SFRP5 is expressed in the RPE and pancreas

Confirming the results of Rattner et al., with murine and rat tissues (15), northern analysis indicated that SFRP2 is highly and preferentially expressed in bovine retina (Fig. 2A). SFRP5 demonstrated a related but distinct expression pattern, showing strong expression in bovine RPE, but minimal, if any, expression in retina, brain, heart, liver, kidney, testis and muscle (Fig. 2B). Human RPE also demonstrated strong expression, although cultured human RPE cells did not show detectable signal (Fig. 2D). Although the reason for this is unclear, it should be noted that
Figure 1. Comparison of the predicted amino acid sequences of murine, bovine and human SFRP5 with murine SFRP1, SFRP2, SFRP3, SFRP4 and frizzled-7.

- residues that are identical to murine SFRP5; [ ] sites at which a space was introduced for purposes of alignment; *, positions of the conserved cysteine residues. The alignment was generated using GeneWorks 2.5.1 (Oxford Molecular Group, Oxford, UK) and modified manually to maximize homology.

cultured cells are often relatively de-differentiated compared with their tissue of origin and, thus, sometimes do not express some differentiated gene products. It is possible that conditions that favor differentiation of cultured RPE cells may lead to induction of SFRP5 expression.

Analysis of non-ocular human tissues revealed significant expression in the pancreas (Fig. 2E). This is consistent with the report of Melkonyan et al. (21), who cloned SARP-3 from the pancreas. On prolonged exposure of the blot, weak SFRP5 expression was also noted in heart, liver and muscle (Fig. 2F), but the level of expression was at least two orders of magnitude lower than that in the pancreas. It should be noted that the intensity of the signals between human RPE and pancreas cannot be compared quantitatively because the RPE lane represents total RNA (10 µg), whereas the pancreas lane represents poly(A) + RNA (2 µg). Nonetheless, it appears that SFRP5 is more highly expressed in the RPE than in the pancreas.

In order to define further the cellular expression pattern of SFRP2 within the retina, we performed in situ hybridization of retinal sections with digoxigenin-labeled probes. With mouse retina and human SFRP2 probe, no signal was detected (data not shown). With bovine retina and human SFRP2 probe, a strong signal was observed throughout the inner nuclear layer (Fig. 3). The reason for the difference in results between the murine and bovine tissues is unclear. Efforts to perform in situ hybridization with SFRP5 probes with murine, rat and bovine retinal sections were not successful due to problems with background staining.

**Human SFRP2 maps to 4q31.3; SFRP5 maps to 10q24.1**

Using radiation hybrid analysis, we mapped human SFRP2 to chromosome 4q31.3, which is syntenic with the previously mapped location of murine SFRP2 in the central region of murine chromosome 3 (15). Somatic cell hybrid analysis localized SFRP5 to human chromosome 10. Radiation hybrid analysis with two sets of primers provided a map location of 10q42.1. This was confirmed by fluorescence in situ hybridization (FISH) analysis that localized SFRP5 to the long arm of human chromosome 10 on both homologs. In the 20 metaphase spreads that were analyzed, a consistent signal was not seen on any of the other chromosomes. A map location of 10q24.1 was assigned by FISH to human prometaphase chromosomes with simultaneous 4′,6-diamidino-2-phenylindole (DAPI) banding (Fig. 4).

**Genomic structure of murine and human SFRP5**

Human and murine genomic SFRP5 clones were obtained by screening human and murine 129/SvJ genomic libraries. The genomic structure for mouse and human SFRP5 was determined (Fig. 5). Both genes contain three coding exons with identical splice sites. All intron–exon boundaries demonstrate the conserved GT–AG splice sequence (26).
Figure 2. SFRP2 is expressed in the retina; SFRP5 is expressed in the RPE and pancreas. (A–C) Northern blot analysis of total RNA (10 µg/lane) extracted from bovine retina, RPE and other tissues. (A) A blot hybridized with a human SFRP2 probe. (B) The same blot hybridized with a bovine SFRP5 probe. (C) The same blot hybridized with a human 18S rRNA probe. (D) Northern blot analysis of total RNA (10 µg/lane) extracted from human eye cup (predominantly RPE) and from the ARPE-19, JRPE, HRPE 156 and HRPE 142 human RPE cell lines, hybridized with bovine SFRP5 probe, and exposed for ∼16 h. (E) Northern blot analysis of poly(A)+ RNA (2 µg/lane) extracted from the indicated human tissues, hybridized with a bovine SFRP5 probe, and exposed for ∼16 h. (F) The same blot as in (E) exposed for 1 week.

Figure 3. SFRP2 is expressed in the inner nuclear layer. (A) Retina frozen section stained with 0.1% thionin. (B and C) In situ hybridization of bovine retina frozen sections with human SFRP2 antisense (B) and sense (C) probes. RPE, retinal pigment epithelium; OS, photoreceptor outer segments; IS, photoreceptor inner segments; ONL, photoreceptor outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

SFRP5 inhibits the ability of Xwnt-8 to induce axis duplication in Xenopus embryos

Overexpression of Xwnt-8 on the ventral side of the Xenopus embryo causes axis duplication (27), and this system has been used extensively to assess elements in the Wnt signaling pathway. To assess the ability of SFRP5 to modulate Wnt signal transduction, we tested whether microinjected SFRP5 mRNA could inhibit Xwnt-8’s ability to induce axis duplication. Ventral vegetal blastomeres at the 32-cell stage were injected with Xwnt-8 mRNA with or without SFRP5 mRNA. Under the conditions tested, SFRP5 significantly reduced the frequency of axis duplication from 96 to 34% (Table 2; Fig. 6). Even among the 34% of SFRP5/Xwnt-8 co-injected embryos that showed evidence of axis duplication, none showed the complete secondary axis formation, including heads and eyes, that is characteristic of embryos injected with Xwnt-8 mRNA alone. This effect was specific to SFRP5, as co-injection of green fluorescent protein (GFP) mRNA did not interfere with Xwnt-8’s induction of secondary axes. Thus, SFRP5 can function to inhibit Wnt signaling.

DISCUSSION

An increasing number of examples are being identified in which soluble polypeptides identical or homologous to the extracellular domains of transmembrane receptors serve to modulate signal transduction (28,29). High levels of soluble fibroblast growth factor (FGF) receptors have been demonstrated in the retina and vitrous and have been postulated to modulate FGF in the eye (30–32). The SFRPs appear to serve this function in Wnt signaling. Unlike some other systems in which splicing is used to generate the soluble form of the receptor, the SFRPs are encoded by genes that are distinct from those for the membrane receptor.

The role of SFRPs in retinal development and function is unknown. Here, we have begun to address these issues. Thus far, chromosomal mapping of the SFRP genes has not provided any functional insight because the genes are not near any known human disease loci or mouse mutations that involve the eye. It should be mentioned that our map location for human SFRP5, 10q24.1, is different from the somatic cell hybrid-derived chromosome 15 localization reported by Melkonyan et al. for SARP-3 (21), which, as noted above, is identical to SFRP5. The basis for this discrepancy is unclear, although it should be noted that we used three independent methods (FISH, somatic cell hybrids and radiation hybrids with two different panels and two different sets of primers) and all three yielded consistent results.
The current model of SFRP action envisages soluble SFRPs acting to modulate Wnt signaling by competitively inhibiting ligand binding to cell surface receptors. Consistent with such a mechanism, SFRP3/Frzb-1’s inhibition of Xwnt-8’s ability to induce axis duplication in Xenopus is not cell-autonomous. SFRP3/Frzb-1 mRNA inhibited Xwnt-8 activity not only when it was co-injected into the same cells as the Xwnt-8 mRNA, but also when it was injected into neighboring cells (16). Our studies demonstrating that SFRP5 also can inhibit axis duplication provide further evidence that SFRPs as a class act to modulate Wnt signaling. This is perhaps somewhat surprising because, as shown in Figure 1, the degree of sequence conservation between the different known SFRPs is relatively low. It is also possible, however, that SFRP5 and/or other SFRPs may act by additional mechanisms that do not involve Wnt signal transduction.

The suggestion that SFRP5 is involved in apoptosis (21) may provide a hint regarding its function in the eye. Neuronal, including photoreceptor, apoptosis is important both during retinal development (33,34) and in retinal diseases such as retinal degeneration (35,36) and glaucoma (37). Thus, it will be important to determine if SFRP5 acts to modulate retinal apoptosis. Such a postulated activity could be mediated through Wnt signaling, especially given the recent finding that perturbation of genes acting downstream of Wnt/wingless, for example D-APC and armadillo, can cause apoptotic photoreceptor cell death in Drosophila (38). Conversely, reduction in Drosophila armadillo can prevent apoptosis in the D-APC mutant, while armadillo overexpression can mimic D-APC inactivation. Furthermore, potentially related to the expression of SFRP5 by RPE cells, there is an association between some human and murine APC gene mutations and the development of congenital hypertrophy of the RPE (39–41).
Figure 6. (A) Lateral view of a stage 35–36 embryo showing a complete secondary axis produced when 20 pg of Xwnt-8 mRNA was injected into a ventral vegetal blastomere. (B) Dorsal view of the embryo in (A), showing the complete secondary axis. (C) Lateral view of a stage 35–36 embryo in which 150 pg of SFRP5 mRNA was co-injected with 20 pg of Xwnt-8 mRNA into a ventral vegetal blastomere. The formation of a secondary axis by Xwnt-8 was inhibited. (D) Dorsal view of the embryo shown in (C).

Table 2. sFRP-5 mRNA inhibits Xwnt-8-induced secondary axis formation

<table>
<thead>
<tr>
<th>mRNA injected</th>
<th>Amount of RNA injected (pg)</th>
<th>No. of animals injected</th>
<th>No. of embryos with a secondary axis</th>
<th>% of animals with a secondary axis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xwnt-8</td>
<td>20</td>
<td>43</td>
<td>41</td>
<td>95</td>
</tr>
<tr>
<td>Xwnt-8</td>
<td>20</td>
<td>26</td>
<td>25</td>
<td>96</td>
</tr>
<tr>
<td>GFP</td>
<td>500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xwnt-8</td>
<td>20</td>
<td>41</td>
<td>14</td>
<td>34*</td>
</tr>
<tr>
<td>SFRP5</td>
<td>100–400</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SFRP5</td>
<td>150</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GFP</td>
<td>500</td>
<td>22</td>
<td>0</td>
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</tr>
</tbody>
</table>

*Significantly different from embryos injected only with Xwnt-8 mRNA (P < 0.05).

The expression pattern of the SFRPs may provide another clue about their function. Although many of the details need to be worked out, and there appear to be some species differences, the SFRPs show specific and distinct expression patterns. For example, SFRP2 is highly expressed in the retina, and less highly expressed in the heart and lung (15). SFRP1 and SFRP3 are also expressed in the retina, but are more widely expressed in other tissues. As defined by in situ hybridization, within the mouse brain, SFRP1 is expressed in the choroid plexus. Within the eye, the predominant signal was noted in the ciliary body and the anterior epithelium of the lens. SFRP4, which was identified from a genomic library, was not found to be highly expressed in any of the tissues tested. SFRP5 is highly expressed in the RPE, and moderately expressed in the pancreas. Whether or not the SFRPs also show functional differences is unclear, but their structural differences and highly regulated expression patterns make this seem likely.

The retina demonstrates a high degree of polarity at both the tissue and cellular levels (42). The photoreceptor cell is both
functionally and morphologically amongst the most highly polarized cells in the body. In *Drosophila*, the Wnt signaling pathway has been implicated in the development of photoreceptor and retinal polarity (43,44). The data described herein suggest that photoreceptors are bathed by two complementary gradients of signaling molecules/modulators, SFRP2 originating from the inner nuclear layer and SFRP5 from the RPE. Although little is known about the specific role of Wnt/frizzled signaling in the vertebrate retina, and even less about the SFRPs, given the involvement of the Wnt/frizzled pathway in polarity determination, it is tempting to speculate that these putative inverse gradients of SFRP2 and SFRP5 may be involved in determining the polarity of photoreceptors and perhaps other cells in the retina. Future experiments will be needed to test this hypothesis.

**MATERIALS AND METHODS**

**cDNA and genomic clones**

A subtracted bovine RPE/retina cDNA library in Uni-ZAP XR (Stratagene, La Jolla, CA) was generated and partially characterized as previously described (25; I.T. Chang et al., in preparation). Using the partial length *SFRP5* cDNA probe obtained from this library as probe, the original pre-subtracted bovine RPE/retina cDNA library was screened by standard methods to obtain full-length clones (45). A human RPE/retina cDNA library in Uni-ZAP XR was screened similarly to obtain human *SFRP5* cDNA clones. A murine 129/Sv/J genomic library in λ-FIX II (Stratagene) (a kind gift of Dr Se-Jin Lee, Johns Hopkins University) was screened to obtain mouse genomic clones. The mouse cDNA sequence was derived from the genomic sequence by comparison with the bovine and human cDNA sequences. Human genomic clones were obtained by screening a high density array of P1 clones according to the manufacturer’s directions (Genome Systems, St Louis, MO). To obtain the full-length murine *SFRP4*-coding region, since the previously reported sequence was only partial length (15), the murine *SFRP4* EST (accession no. AA087277) was obtained and sequenced.

**Northern blot hybridization**

Northern blot hybridization was performed as previously described (42,46). Total RNA was prepared by homogenization of bovine tissues in guanidinium thiocyanate (47). Aliquots (10 µg) of total RNA were loaded onto a 1% agarose gel in the presence of formaldehyde. After transfer to a nylon membrane, the RNA was hybridized with a probe generated either from the whole *SFRP5* cDNA, or from the *Pst*I fragment of human *SFRP2*. For human northern blot analysis, human total RNAs from *in vivo* RPE and RPE cell cultures (48,49) were isolated as described above. In addition, a blot containing human poly(A)+ RNAs was obtained from Clontech (Palo Alto, CA). Human northern blots were hybridized with the bovine *SFRP5* probe. Filters were washed twice with 2× SSC/0.1% SDS at room temperature for 5 min, followed by two washes at 68°C with 0.1× SSC/0.1% SDS for 15 min.

**In situ hybridization**

*In situ* hybridization with retinal frozen sections from the non-pigmented region of bovine eye was performed using digoxigenin-labeled antisense and sense RNA probes as described previously (42,46). The antisense and sense probes were generated from an EST clone (accession no. H87071) containing human *SFRP2* cDNA sequences.

**Chromosome mapping**

Radiation hybrid mapping of *SFRP5* was performed using the Standford G3 and GeneBridge 4 panels according to the supplier’s directions (Research Genetics, Huntsville, AL). Two sets of primers were used (the 5′ primes were 5′-ACGAATGTGTTGTAATGGAC-3′ and 5′-GATGTTGGGCTTCCTCCTCA-3′; the same 3′ primer was used for both sets of reactions: 5′-AATGAAATCCCGGAAATCGA-3′). For *SFRP2*, the 5′ primer 5′-ACTGTGACTTGGGCTTGTTG-3′ and 3′ primer 5′-GTGATACATGTAACAGTATTAAAGTTC-3′ were used with the Standford G3 panel. A Chinese hamster–human somatic cell hybrid panel was used according to the supplier’s directions (Oncor, Gaithersburg, MD).

For FISH analysis (50), 1 µg of *SFRP5* human genomic P1 DNA was labeled with digoxigenin (Boehringer Mannheim, Switzerland) in a nick translation reaction (Life Technologies, Gaithersburg, MD). An aliquot (200 ng) of labeled probe was precipitated with 5 µg of human Cot-1 DNA, resuspended in 50% formamide, 2× SSC, 10% dextran sulfate, denatured for 5 min at 75°C, and pre-anealed for 1 h. Slides were prepared from phytohemagglutinin-stimulated human blood lymphocytes by standard methods (51). Slides were denatured for 5 min at 75°C before hybridization at 37°C for 18 h. The slides were washed twice in 50% formamide, 2× SSC and twice in 2× SSC at 42°C. Probe was detected with rhodamine-conjugated anti-digoxigenin antibody according to the manufacturer’s instructions (Oncor). Chromosomes were counterstained with the A-T-binding fluorochrome DAPI and viewed with a Zeiss Axioskop equipped with a SenSys cooled CCD camera (Photometrics, Tucson, AZ) and Smart Capture imaging software (Vysis, Downers Grove, Il).

**Embryo collection and blastomere injection of mRNAs**

Fertilized *Xenopus laevis* eggs were obtained by gonadotropin-induced natural mating of adult frogs, and the jelly coat was removed chemically as previously described (52). Only embryos exhibiting stereotypic cleavages (52–54) were used to identify the ventral vegetal blastomeres across the population of experimental embryos. Embryos were staged according to Nieuwkoop and Faber (55).

Capped and polyadenylated mRNAs for *Xwnt-8* (cloned into the pCS2 vector; a generous gift of Randy Moon, Howard Hughes Institute and University of Washington, Seattle, WA), *SFRP5* (cloned into pCS2 vector) and GFP (cloned into SP64R vector) were in *vitro* transcribed using the Message Machine kit according to the manufacturer’s protocol (Ambion, Austin, TX). A single ventral vegetal blastomere of the 32-cell embryo was microinjected with either 20 pg of *Xwnt-8* mRNA, 20 pg of *Xwnt-8* combined with 500 pg of GFP mRNA, 20 pg of *Xwnt-8* mRNA combined with 100–400 pg of bovine *SFRP5* mRNA, 150 pg of *SFRP5* mRNA, or 500 pg of GFP mRNA in a 1–2 nl volume. During injections, embryos were cultured in 3% Ficoll in 100 mM Steinberg’s solution, and after 1–2 h were raised in 50% Steinberg’s solution until late tail bud (stages 35–36).
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ABBREVIATIONS

CRD, cysteine-rich domain; RPE, retinal pigment epithelium; SFRP, secreted frizzled-related protein.

REFERENCES


