SFRP1 modulates retina cell differentiation through a β-catenin-independent mechanism

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Summary

Secreted frizzled related proteins (SFRPs) are soluble molecules capable of binding WNTS and preventing the activation of their canonical signalling cascade. Here we show that *Sfrp1* contributes to chick retina differentiation with a mechanism that does not involve modifications in the transcriptional activity of β-catenin. Thus, addition of SFRP1 to dissociated retinal cultures or retroviral mediated overexpression of the molecule consistently promoted retinal ganglion and cone photoreceptor cell generation, while decreasing the number of amacrine cells. Measure of the activity of the β-catenin-responsive Tcf-binding site coupled to a luciferase reporter in transiently transfected retinal cells showed that *Sfrp1* was unable to modify the basal β-catenin transcriptional activity of the retina cells. Interestingly, a dominant-negative form of GSK3β gave similar results to those of *Sfrp1*, and a phosphorylation-dependent inhibition of GSK3β activity followed SFRP1 treatment of retina cells. Furthermore, retroviral mediated expression of a dominant-negative form of GSK3β induced a retina phenotype similar to that observed after *Sfrp1* overexpression, suggesting a possible involvement of this kinase in SFRP1 function.

Key words: Chick, Eye development, Retina ganglion cells, Wnt signalling, GSK3β

Introduction

WNT proteins are a large family of cysteine-rich, secreted glycoproteins, associated with the extracellular matrix, that regulate cell fate and cell behaviour in a wide variety of biological processes, including embryonic axis formation, neural development, myogenesis, organogenesis and oncogenesis (Patapoutian and Reichardt, 2000; Wodarz and Nusse, 1998). During nervous system development, Wnt signalling participates in the induction of posterior neural tissue in *Xenopus* and mammalian embryos (Kiecker and Niehrs, 2001). WNTs are also required for midbrain development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990) and for controlling the proliferation of precursor cells of the neural crest, neural tube and hippocampus (Dickinson et al., 1994; Ikeya et al., 1997; Lee et al., 2000b; Megason and McMahon, 2002).

Wnt activities turn on distinct signalling cascades. In all cases, Wnt signalling is initiated by WNT binding to Frizzled (Fz), a family of G-coupled transmembrane receptors (Liu et al., 2001; Winklbauer et al., 2001). In the canonical pathway, receptor-ligand interaction leads to a protein complex-mediated inhibition of the glycogen synthase kinase3β (GSK3β) with a consequent cytoplasmic accumulation of β-catenin, which activates Wnt target genes after nuclear translocation (Wodarz and Nusse, 1998). Alternatively, Fz activation appears either to stimulate intracellular Ca^{2+} release, leading to activation of protein kinase C (Kuhl et al., 2000; Winklbauer et al., 2001), or to activate the planar cell polarity pathway that involves Jun-kinase mediated transcription (Heisenberg et al., 2000; Strutt, 2001; Weber et al., 2000). The complex signalling mechanisms of WNTs are further controlled in the extracellular space by a number of soluble molecules including the family of Secreted Frizzled Related Proteins, SFRPs (Jones and Jomary, 2002)

As their name indicates, SFRPs are secreted proteins that share structural homology with the extracellular cystein rich domain (CRD) of the Fz domain (Finch et al., 1997; Melkonyan et al., 1997; Rattner et al., 1997; Wang et al., 1997), which in Fz is necessary and sufficient for WNT binding (Wodarz and Nusse, 1998). The structural homology of SFRPs with the Wnt receptors and their widespread expression, complementary in several locations to those of *Wnts*, have suggested that SFRPs could act as competitive antagonists of WNT-mediated β-catenin signalling, binding and preventing Wnt interaction with the Fz receptors (Leyns et al., 1997; Wang et al., 1997; Xu et al., 1998). However, both biochemical and functional analyses of SFRP activities have demonstrated that SFRP mode of action might be more elaborate than that originally envisaged. SFRP1 can form heterodimeric complexes with other members of the family or with Fz receptors, suggesting alternative mechanisms of interfering with the Wnt pathway (Bafico et al., 1999). Furthermore, expression studies in *Xenopus* embryos and analysis in mammalian cell lines indicated that different SFRPs have opposing activities (Bradley et al., 2000; Melkonyan et al., 1997; Pera and De Robertis, 2000) and few of them may function independently of any known WNTs, suggesting that SFRPs might regulate developmental processes in an autonomous way (Bradley et al., 2000).

Recently, we and others have isolated and studied the

distribution of the chick homologue of *Sfrp1* (Esteve et al., 2000; Terry et al., 2000). *cSfrp1* has a widespread and dynamic expression in the developing chick embryo and is abundantly localized during the processes of neuronal differentiation in distinct CNS regions, including the eye. Initiation of eye development appears to require the Wnt signalling cascade (Heisenberg et al., 2001; Rasmussen et al., 2001). However the precise role of Wnt, Fz receptors and SFRPs, all expressed in the eye at later stages of development (Deardorff and Klein, 1999; Jasoni et al., 1999; Jin et al., 2002; Sumanas and Ekker, 2001), has yet to be defined.

With in vitro and in vivo studies, we show here that SFRP1 modulates cell differentiation of the chick retina promoting the generation of retinal ganglion cells (RGC) and cone photoreceptor cells, at the same time as decreasing the number of amacrine cells. Because retina cells have a low basal β-catenin transcriptional activity that is not modified by SFRP1, we propose that SFRP1 contributes to retina neurogenesis with a mechanism that does not require its interference with a β-catenin-dependent Wnt-Fz interaction.

Materials and Methods

Chick embryos

Fertilized chick eggs (White Leghorn) were incubated at 38°C and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Isolation of *cSfrp1* cDNAs and in situ hybridisation

cSfrp1 isolation and in situ hybridisation procedures have been previously described (Esteve et al., 2000). The accession number for c*Sfrp1* is AJ404652 in the Nucleotide Sequence Databases.

Expression and purification of chick SFRP1 protein and its derivatives

The coding sequence of *Sfrp1* was cloned in the pCDNA3.1 eukaryotic expression vector (Invitrogen, Carlsbad, CA), containing a myc/his-tag. The resulting construct coded for the SFRP1 protein in frame with the tag in its C-terminus. Subconfluent MDCK cells, grown in Dulbecco´s modified Eagle´s medium (DMEM; Gibco, BRL, Paisley, UK) supplement with 10% FCS were transfected with 5 μg of *Sfrp1* expression plasmid or with the empty vector, using the Gene PORTER kit (Gene Therapy Systems, San Diego, CA). Transfected cells were selected with Geneticine (500 μg/ml) for three weeks. Individual G418-resistant clones were isolated and expression of the recombinant protein was determined by immunoblots with an anti-myc mAb (clone 9E10). The myc/his-SFRP1 was partially purified from conditioned medium (CM) using a Ni-NTA agarose (Qiagen, Valencia, CA) column. CM from MDCK transfected with the empty vector was processed in parallel as control. The agarose was washed and equilibrated in 50 mM phosphate buffer containing 10 mM imidazole. SFRP1 protein was eluted with 100 mM imidazole, as reported (Uren et al., 2000). The control and SFRP1 eluted fractions were analysed by silver staining and western blotting.

Dissociated retinal cell culture

The central portion of the neural retinas from embryonic day (E)5 chick embryos were dissected free of pigmented epithelium and dissociated as described (Bovolenta et al., 1996). Cells were plated in 33 mm dishes coated with poly-D-lysine (20 μg/ml; Sigma) and cultured in DMEM supplemented with N2 nutrients (Gibco BRL) alone or diluted 1:1 with CM from MDCK/control or MDCK/SFRP1. For immunostaining, cultures were fixed in methanol containing 10% DMSO for 15 minutes at room temperature.

dsRNA-mediated interference

The templates used for RNA synthesis were either a linearised plasmid containing hGFP, as a control, or the products of PCR reactions using primers designed to amplify the N-terminal (nucleotide 75 to 410) or the C-terminal (nucleotide 506 to 901) regions of chick *Sfrp1*. The products were purified using GFX PCR DNA and Gel Purification Kit (Amersham Pharmacia, Buckinghamshire, UK). Each primer contained a T7 promoter sequence on its 5′ end such that sense and anti-sense RNAs, in an already annealed conformation, can be synthesized simultaneously from a single PCR-derived template using T7 RNA polymerase (Kennerdell and Carthew, 1998). Following removal of template with DNAseI, RNAs were purified using RNeasy Mini Kit (Qiagen). dsGFP RNA was generated from ssRNAs synthesized using T7 and SP6 polymerase. The sense and anti-sense GFP RNAs were mixed in annealing buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA) heated at 68°C for 10 minutes and incubated at 37°C for 4 hours. The specificity of the dsRNA, designed against the N-terminal or C-terminal portion of *Sfrp1*, was tested using MDCK cells transfected with myc-*Sfrp1*. The three dsRNAs were either electroporated or added to the dissociated cell suspension (2.5 μg/6×10^5 cells). After incubation cells were seeded, cultured for 24 hours and harvested. The ability of *Sfrp1* dsRNAs to interfere with myc-Sfrp1 mRNA and protein levels were determined by PCR analysis and western blot of the CM of control and dsRNA treated cells using anti-myc mAb. The Cter *Sfrp1* dsRNA resulted the most effective and was used in the experiments involving retina cells. In each experiment, the level of endogenous retina *Sfrp1* mRNA was monitored in dsRNA treated, control treated and untreated cultures using time-course PCR analysis. In all experiments, *Sfrp1* Cterm dsRNA reduced the mRNA level to about 60% of controls.

Reporter assays

E5 dissociated central retinal cells, prepared as described above, were seeded in 24-well plates and transfected 3 hours later using the Gene PORTER kit (Gene Therapy Systems). In each case the 2.5 μg/well of total DNA contained 200 ng of a plasmid constituted by a Lef-1 responsive luciferase reporter composed of four copies of the wild-type Lef-1 responsive element (or the mutated element) upstream of the prolactine minimal promoter driving luciferase expression (generated and kindly provided by Drs Gutierrez and M.O. Landázuri, Hospital de la Princesa, Madrid) and 50 ng of pRL-TK (Promega, Madison, WI) together with variable amounts of the effector plasmids or the empty vector. Twenty-four hours after the transfection, the luciferase activities were determined using a dual-luciferase assay system (Promega). The LEF-1 reporter luciferase activity was normalized with *Renilla* luciferase activity to account for transfection efficiency. The experiment was repeated four times in duplicate.

Explant electroporation

Retina explants from E5 central retinas were suspended in PBS containing 100 ng/μl of DNA (pCSII-h*Gfp* of pCDNA3.1-*Sfrp1*) and electroporated with the following conditions: four pulses of 50 milliseconds length, 508 milliseconds frequency, 20 V. After electroporation explants were grown in suspension for 13 hours in DMEM/N2. Timing of initial protein expression was determined by appearance of GFP fluorescence using a Leica, MZ-FLIII macroscope.

Western blotting of dissociated retina cells or explant culture

Retina cultures were collected at 20 minutes, 3, 5 and 24 hours in PBS. Explants were harvested approx 5 hours after detection of GFP

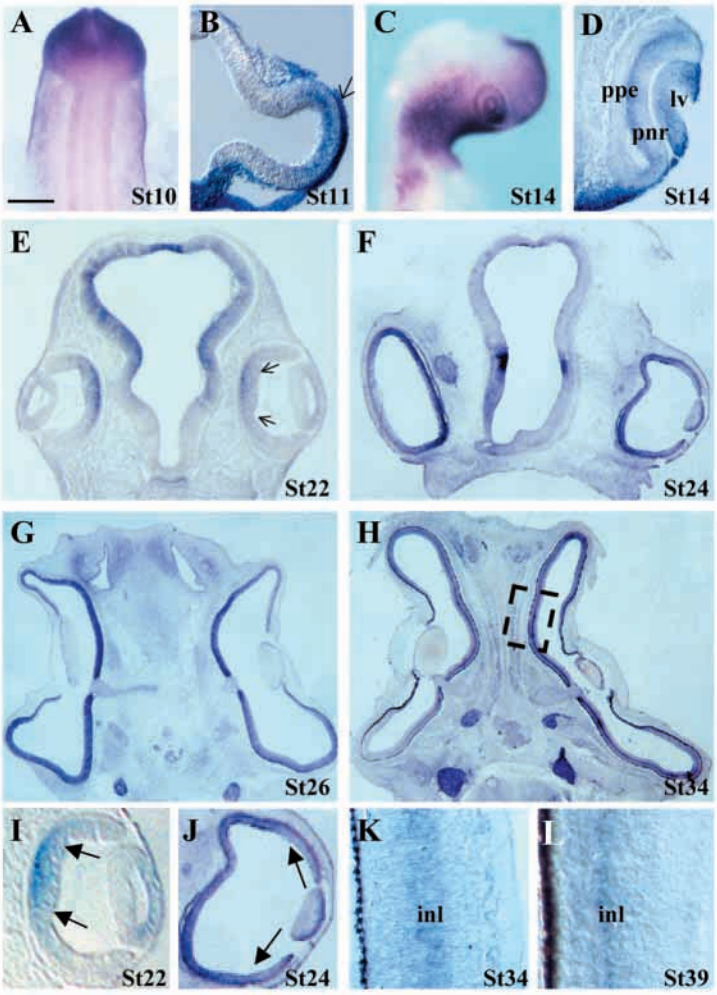

Fig. 1. Localization of chick *Sfrp1* transcripts during eye development. Embryos of indicated developmental stages were hybridised (A-E) in toto or (F-H,K,L) in frontal cryostat tissue sections. Embryos are viewed (A) dorsally or (C) laterally. Images in (B,D,E) represent (B) transversal and (D,E) frontal paraffin sections of whole mount processed embryos. Image in (I,J,K) are high magnification views of (E,F) and of the boxed area in (H), respectively. Note the early expression of *Sfrp1* in the prospective neural retina, (arrow in B) lens placode ectoderm and (A-D) lens vesicle. *cSfrp1* transcripts are first localized to (arrows in E,I) the centro-dorsal retina, expand later to (F,G,J) the periphery and become restricted to (H,K,L) the inl following neurogenesis gradients. inl, inner nuclear layer; lv, lens vesicle; pnr, presumptive neural retina; ppe, presumptive pigment epithelium. Bar: (A,F,G) 400 μm; (B) 94 μm; (C) 500 μm; (D,E,G) 50 μm; (K,L) 40 μm; (H) 625 μm.

expression. Tissue was lysed in a buffer composed of 15 mM Tris, pH 8, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 1% Nondiet-P40, 1 mM Na orthovanadate, 100 mM NaF and proteases inhibitors. Samples were boiled in Laemmli's buffer and separated by SDS-PAGE, transferred to nitrocellulose and probed with the following antiserum: phospho-GSK3β (Ser9; Cell Signalling Technology, Beverly, MA), phospho-GSK3β (Tyr216; Cell Signalling Technology) and α-tubulin (Sigma) or with a mAb anti-GSK3β (Transduction Laboratories, Exeter, UK). For detection of cytosolic β-catenin levels, cells were harvested by lysis in hypotonic solution (10 mM Tris buffer, pH 7.4; 200 μM $MgCl_2$, 1 mM PMSF, 10 μg/ml leupeptine and 10 μg/ml aprotinine). Membrane and cytosolic fractions were separated by ultracentrifugation at 100,000 ***g*** for 30 minutes. Nitrocellulose membranes of the fractionated samples were obtained as above and probed with anti-β-catenin mAb (Transduction Laboratories). Immunoreactive bands were detected with the ECL system (Amersham-Pharmacia).

Retrovirus production and embryo infections

c*Sfrp1* or the dominant-negative form of the *Xenopus* GSK3β [dnGSK3β; K85R abolishing kinase activity (Dominguez et al., 1995)] were cloned into the RCAS(B) vector and viruses were prepared as described (Fekete and Cepko, 1993). Alkaline phosphatase-RCAS(B) viruses were used as control. Concentrated viral suspensions (approx 10^8 cfu/ml) were injected into the optic vesicles of stage 10-11 embryos. Embryos were harvested at E6 or E10, fixed in 4% paraformaldehyde and sectioned on a cryostat at 16 μm of thickness.

Immunohistochemistry

Staining of cultures and tissue sections was performed following standard procedures. The antibodies used are mAb anti-tubulinβ-III (Tuj-1, MEDPASS S.a.r.l., Gran Duché de Luxembourg), rabbit polyclonal anti-phospho-histone 3 (Upstate Biotechnology, Lake Placid, NY), a mitosis marker (Mahadevan et al., 1991), mAb anti-neurofilament 145 kDa protein (CHEMICON, Ternecula, CA), anti-Brn3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-cellular retinoic acid protein 1 (CRABP1; Affinity Bioreagents, Golden, CO) and anti-islet1 guinea-pig antiserum (gift of T. Jessell, Columbia University, NY). The mAbs anti islet-1, anti-Pax6, and anti-visinin developed by T. Jessell, A. Kawakami and C. Cepko, respectively, were obtained by the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA).

For cell culture staining, secondary antibodies were biotin-conjugated anti-mouse or anti-rabbit IgG followed by Alexa-594- or Alexa-488-conjugated streptavidin (Molecular Probes, Eugene, OR). For tissue section staining, biotinylated-secondary antibodies were followed by peroxidase-coupled streptavidin (Jackson Laboratories, West Grove, PA) and AEC system chromogenic reaction (Lab Vision Corporation, Fremont, CA). Cultures were counterstained with BisBenzimide (HOESCHT no. 33342; Sigma).

Statistical analysis

Quantitative data were obtained by counting in each case the number of Hoescht and immuno-positive cells present in a field of 0.038 mm^2. Each experiment was performed in duplicate at least four times; in each case 12 randomly chosen fields were analysed. Data are expressed as mean±s.e.m. The significance of the differences among groups was evaluated by unpaired Student's *t*-test (GraphPad Prism).

Results

Expression pattern of *Sfrp1* during eye development

The chick *Sfrp1* was isolated from a cDNA subtraction library as described previously (Esteve et al., 2000). Northern blot analysis of embryos at different developmental stages revealed a mayor transcript of 4.9 kb (not shown). *Sfrp1* has a widespread and dynamic expression in the developing chick embryo with high levels in the anterior neural plate including the eye anlage (Esteve et al., 2000; Terry et al., 2000). *Sfrp1* transcripts are expressed throughout embryonic development of the eye (Fig. 1). In the optic vesicle and optic cup, *Sfrp1* expression was found in the prospective lens placode ectoderm and in the lens vesicle as well as polarized to the ventricular

surface of the prospective neural retina but not of the prospective pigment epithelium (Fig. 1A-D), where its expression was detected only at later stages (Fig. 1K). After optic cup formation, cells in the neural retina begin to exit the cell cycle and progressively differentiate following a centro-peripheral, dorso-ventral and temporo-nasal gradient (Prada et al., 1991). *Sfrp1* mRNA distribution paralleled this gradient: it was strongly localized to the centro-dorsal region of the retina at stage 22 (Fig. 1E,I) and expanded to the periphery at later stages (Fig. 1F,G,J). As neurons accumulate in their respective layers, expression of *Sfrp1* became restricted to the inner nuclear layer of the retina (Fig. 1H,K,L), where the last neurons are born. This expression pattern was consistent with the hypothesis that SFRP1 could participate in neural retina differentiation.

Production and purification of secreted SFRP1

To address the functional activity of SFRP1, we generated a source of soluble SFRP1 protein. The MDCK cell line was stably transfected with an expression vector containing the full coding sequence of myc-tagged *Sfrp1* or with the vector alone. To optimise the production of SFRP1, single-cell-originated clones were isolated and tested for their ability to secret the tagged protein. Although SFRP1-myc was detected in the cell line extracts (not shown), the majority of the protein was released in the culture medium of several of the isolated clones, as shown by immunoblots (Fig. 2A). Clones 2, 3 and 10 secreted the highest yields of SFRP1 into the media and were used in the subsequent studies (MDCK/SFRP1). The culture media from cells transfected with the empty vector or media from clones unable to secrete the protein (i.e. clone 7, Fig. 2A) were used as controls (MDCK/control).

Purification of SFRP1 from the conditioned medium (CM) was attempted using an affinity chromatography on a Ni^{2+} charged agarose column. This single step procedure provided only a partial purification of the protein as assessed by silver staining of the recovered fraction and comparison with MDCK/control CM processed in parallel (Fig. 2B). An additional purification step on a heparin-agarose column, as previously described (Uren et al., 2000), did not improve the protein purification when compared with the control (not shown). The CM or the affinity semi-purified protein were used in subsequent studies aimed at defining the activity of SFRP1 during chick neural retina development.

SFRP1 induces differentiation of retinal neuroepithelial cells

Initial functional studies were performed on dissociated retinal cultures, where the direct response to SFRP1 could be assessed on each individual cell. The order of retina cell type generation is conserved in vertebrates (Livesey and Cepko, 2001), and retinal ganglion cells (RGC) and cone photoreceptors constitute the postmitotic neurons present in an E5 chick retina (Prada et al., 1991). The majority of the retina neuroepithelium is still undifferentiated and precursor cells can proliferate and differentiate in culture. Thus, E5 retinas were dissociated, plated on poly-D-lysine (PDL)-coated dishes and maintained in defined medium for 24 hours. Under these conditions and compared with control cultures grown in the presence of CM

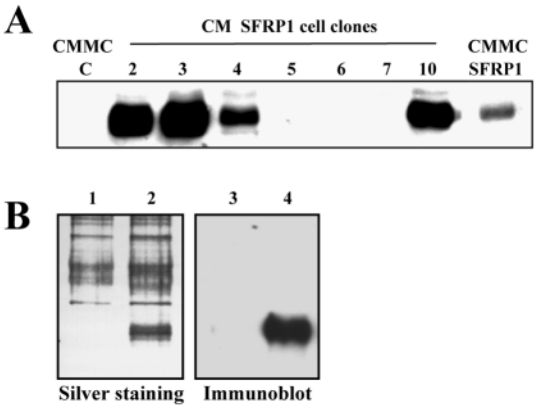

Fig. 2. Generation and purification of recombinant and soluble SFRP1. (A) The conditioned media (CM) from MDCK/control or MDCK/SFRP1 mass cultures (MC) and few of the derived clones were tested for the presence of secreted SFRP1 by immunoblot with anti-myc antibody. Clones 2, 3 and 10 secreted the highest yield of SFRP1 compared with the yield from MC or clone 4. (B) Partial purification of SFRP1. Concentrated CM from either clone 10 [see (A)] or MDCK/control were applied to Ni^{++}-NTA agarose column and eluted with 100 mM imidazole. Eluted fractions were separated by 10% SDS-PAGE. The protein content of a representative fraction eluted from control (lanes 1 and 3) or clone 10 (lanes 2 and 4) media were compared. The identity of SFRP1 in the fraction was confirmed by immunoblot with anti-myc antibody.

from MDCK/control, addition of MDCK/SFRP1 CM or its semi-purified fraction did not induce significant variations in cell density (Fig. 3G) or in the rate of apoptotic cell death (not shown) and mitotic cell division, as determined by staining with the mitotic marker PH3 (Fig. 3G). However, SFRP1-treated cultures showed an increase in cell differentiation, as assessed by staining with antibodies against distinct differentiation markers: islet1/2, LIM-domain containing proteins, expressed in the retina predominantly by RGC (Austin et al., 1995), visinin, expressed by cone photoreceptors (Hatakenaka et al., 1985), and two other general neuronal markers, the 145 kDa neurofilament (NF) protein and Tuj1, a neuron-specific tubulinβIII, which is expressed soon after the last mitotic division. Thus, in the presence of SFRP1, there was an increase in the number of Tuj1- (Fig. 3G), NF- (Fig. 3A,B) and visinin-positive cells (Fig. 3E-G) with respect to controls. This difference was less pronounced when the number of differentiated cells was determined by staining with anti-islet1/2 mAb (Fig. 3C,D,G), possibly reflecting the later onset of this marker in the process of differentiation, as compared with Tuj1. Alternatively, other phenotypes may be included in the pool of Tuj1-positive cells.

At E5, Sfrp1 is endogenously expressed in most of the retina central neuroepithelium. Therefore, interference with its expression should at least in part modify the basal cell differentiation normally occurring in the control cultures. We employed double-strand (ds) RNA-mediated interference (RNAi) to deplete endogenous SFRP1 (Kennerdell and Carthew, 1998). To assess the specificity of the designed dsRNA, dsRNA directed against the N-terminal or C-terminal portion of *Sfrp1* and a control dsRNA against GFP were added to MDCK cells transfected with myc-*Sfrp1*. The three dsRNAs

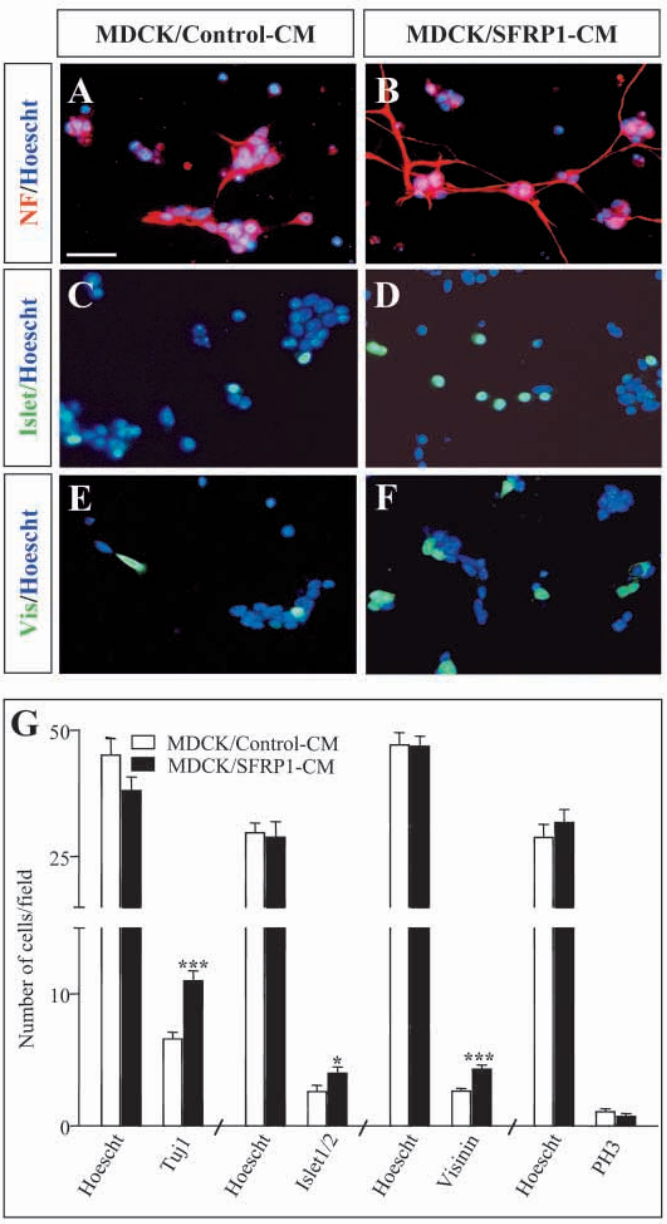

Fig. 3. SFRP1 affects cell differentiation in retina cell cultures. E5 chick retinas were dissociated and grown for 24 hours in the presence of CM from (A,C,E) MDCK/control or (B,D,F) MDCK/SFRP1. After fixation, cultures immuno-stained with antibodies against (A,B) anti-NF, (C,D) islet1/2, (E,F) visinin or phospho-histone3 (PH3, a mitotic marker) and counterstained with Hoescht (blue staining). (G) Final cell density (Hoescht$^+$), the degree of differentiation (Tuj1$^+$; islet1/2$^+$; visinin$^+$) and mitotic rate (PH3$^+$) were evaluated in control (empty bars) and treated cultures (filled bars) by counting, in each experimental condition, the number of immuno-positive cells and the total number of cells in 14 randomly selected microscopic fields. Data represent a typical experiment performed in triplicate. Note how the presence of SFRP1 increases the number of differentiated cells without significant variations in the total number of cells or in the rate of cell division. *P<0.05; ***P<0.0001, Student's t-test. Bar, 185 μm.

were either electroporated or added to the dissociated cell suspension. Cells were then seeded and harvested after 24 hours. The ability of *Sfrp1* dsRNAs to interfere with myc-SFRP1 protein levels was determined by western blotting of the CM of control and dsRNA treated cells using anti-myc antibodies. The Cter *Sfrp1* dsRNA, and to a lower extent the Nter *Sfrp1* dsRNA, but not the *Gfp* dsRNA, consistently reduced the protein level (30% reduction) of *Sfrp1* in the CM of treated cells (Fig. 4A), without affecting cell viability. PCR analysis showed that the decrease in protein levels induced by the *Sfrp1* Cter dsRNA was paralleled by a clear reduction in the mRNA levels (Fig. 4B). This measure was subsequently used to analyse the interference with the endogenous expression of *Sfrp1* in E5 retinal cells.

Cter *Sfrp1* dsRNA significantly diminished the endogenous mRNA level of *Sfrp1* (Fig. 4C) and interfered with the differentiation of both islet1/2-positive RGC and visinin-positive cone photoreceptors when compared with that observed in the presence of a control dsRNA (Fig. 4D), which effect, in turn, was undistinguishable from that of untreated cultures (data not shown). In no cases was modification in the viability of the cultures ever observed.

Altogether these data are consistent with the idea that SFRP1 specifically favours retina cell differentiation.

Specific neuron classes are altered by overexpression of SFRP1 in ovo

Neuronal differentiation is inhibited by the Delta-Notch signalling pathway, which requires cell-cell contact (Artavanis-Tsakonas et al., 1995). Therefore, we asked whether forcing the expression of SFRP1 in intact chick retinas had consequences similar to those observed in dissociated cells. The replication competent retroviral vector RCAS was used to overexpress SFRP1 in the optic vesicles of St10-11 embryos, when the entire neuroepithelium is still undergoing proliferation. Embryos were allowed to develop until E6, when the majority of RGC, amacrine and cone photoreceptor cells have exit the cell cycle (Prada et al., 1991). Using cell-specific differentiation markers, we analysed whether the number of RGC (islet1-positive), cone photoreceptors (visinin–positive) and amacrine cells [CRABP1-positive (Henrique et al., 1997)] was modified in viral infected retina tissue (Fig. 5A,B). Immunostaining of consecutive sections clearly indicated that RCAS-Sfrp1 infection caused an increase in the number of cones (Fig. 5G,H). Similarly, RGC were not only more densely packed in the RGC layer but were also more frequent in the ventricular zone, as compared with equivalent retina regions of uninfected contralateral eyes or of eyes from RCAS-AP infected embryos (Fig. 5E,F). In RCAS-SFRP1 treated retinas, a larger number of migrating neurons was also observed with antibodies against Tuj1 (Fig. 5C,D). On the contrary, CRABP1-positive amacrine cells, which are generated slightly later than RGC and cones, were less abundant than in controls (Fig. 5I,J). Comparison of the amount of islet1-, visinin- and CRABP1-positive cells in patches of infected retinas with equivalent control regions demonstrated an increase of about 30 to 40% in both RGC and cones, paralleled by a significant decrease in amacrine cells (Table 1A). Similar variations were observed also at later stages of retina development (E10), using additional differentiation markers such as Brn3 or Pax6 (Hitchcock et al., 1996; Liu et al., 2000).

In conclusion, SFRP1 overexpression favoured the

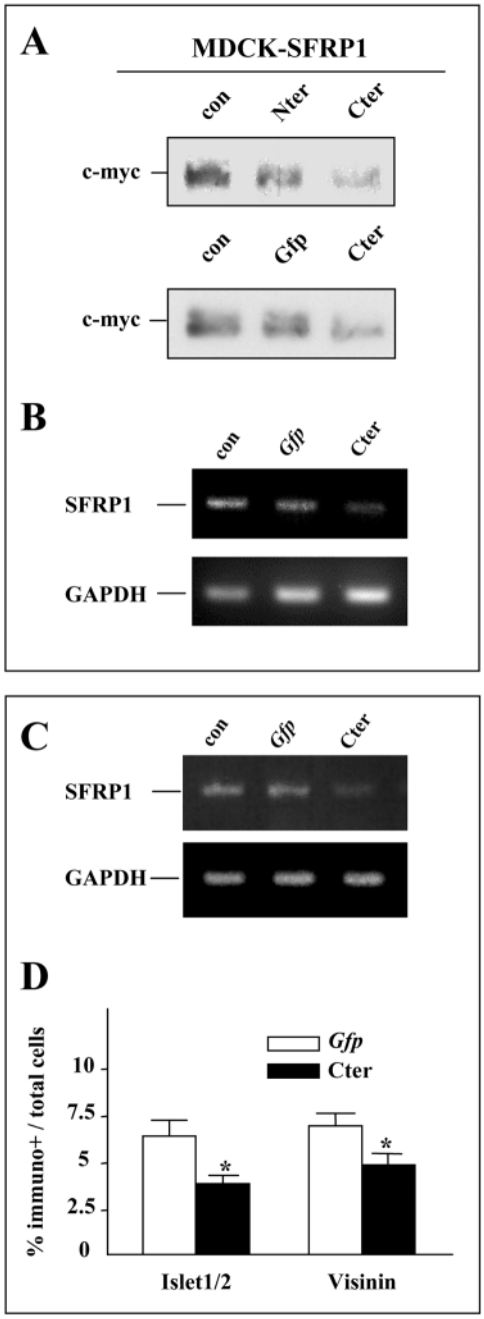

Fig. 4. DsRNA-mediated interference of endogenous *Sfrp1* reduces cell differentiation. (A) To assess specificity of the designed dsRNAs, dsRNAs were introduced into MDCK/SFRP1. The ability of *Sfrp1* dsRNAs to interfere with myc-SFRP1 protein levels was determined by western blots of the CM of control (con) and dsRNA treated cells using anti-myc antibodies. The Cterm *Sfrp1* dsRNA [Cter, two independent evaluations in the upper and lower panels in (A)], and to a lower extent the Nterm *Sfrp1* dsRNA (Nter) but not the *Gfp* dsRNA (Gfp), reduced the protein level in the CM of treated cells. (B) Similar reduction was observed in the mRNA content as determined by RT-PCR analysis. (C) RT-PCR showing chick mRNA levels of *Sfrp1* in untreated, control dsRNA (*Gfp*) or *Sfrp1* dsRNA (Cterm) treated retina cells. Cterm *Sfrp1* dsRNA induced a decrease in the endogenous level of *Sfrp1* mRNA. (B,C) *Gapdh* amplifications were used as controls. (D) E5 dissociated retina cultures were treated with dsRNAs and cultured for 24 hours. Cultures were immunostained with antibodies against islet1/2, or visinin and counterstained with Hoescht. The presence of Cterm *Sfrp1* dsRNA decreases the number of both islet1/2 and visinin-positive cells. **P*<0.05; ***P*<0.001, Student's *t*-test.

production of RGC and cones, while decreasing the number of amacrine cells. These changes occurred without apparent modifications in the rate of apoptotic cell death (data not shown) or cell division, as determined by staining with the mitotic marker PH3 (Fig. 5K,L, and Table 1), or in the mRNA distribution of either *Notch* or *Delta1* (data not shown). Infection with RCAS-AP, used to determine possible unspecific effect of the virus, did not alter retina differentiation as compared with uninfected controls.

Analysis of RCAS-SFRP1 infected embryos at the beginning of retina differentiation (E3-4) already showed an increase of both Tuj1 or islet1 positive cells in the central retina. However, infections in the retina periphery, where the wave of cell differentiation had not spread yet, did not induce the appearance of ectopic neurons, indicating that SFRP1 overexpression can modify cell differentiation but, on its own, cannot initiate it.

SFRP1-mediated effects do not involve modification in the β-catenin-mediated transcriptional activity

WNT-mediated cell differentiation signals through the canonical β-catenin-dependent pathway (Miller et al., 1999; Patapoutian and Reichardt, 2000). SFRP1-induced phenotype in the retina could be the result of SFRP1 interference with an endogenous Wnt-Fz interaction that signals through the β-catenin pathway. If this is the case, then, SFRP1 should modify the cytosolic β-catenin levels and the β-catenin-dependent transcriptional activity of the retina. However, time-course immunoblot analysis of the retina cells cultured in the presence or absence of SFRP1 did not reveal significant variations in the content of cytosolic β-catenin (Fig. 6A), even 24 hours later (data not shown). We next measured the activity of the β-catenin-responsive Tcf-binding site (or of its mutated version) coupled to the luciferase reporter gene in transiently transfected retinal cells. This assay showed that chick retina cells have a low basal β-catenin-dependent transcriptional activity, since similar values were obtained with both the wild type and the mutated version of the reporter plasmid, in line with a recent report in zebrafish retina (Dorsky et al., 2002). Furthermore, transfections of different doses of *Sfrp1* were unable to modulate this basal TCF-transcriptional activity. Nevertheless, *Sfrp1* was able to decrease in a dose-dependent manner the 240-fold activation of the TCF-dependent transcriptional activity induced by *Wnt8* (Fig. 6B). These results suggest that, in developing chick retina, canonical Wnt signalling is not normally activated and, therefore, in the absence of β-catenin-dependent Wnt signalling, SFRP1 may exert its function with an alternative mechanism.

GSK3β might be an effector of SFRP1 activity in the chick retina

The current model of the canonical Wnt signalling suggests

Table 1

(A) Percentage of cell types generated in the E6 retinas infected with RCAS-Sfrp1

E6 central retina	Ganglion cells (islet1/2$^+$)	Cone photor (visinin$^+$)	Amacrine cells (CRABP1$^+$)	Mitotic cells (P-H3$^+$)
Control	35.7±2.2 **100%** (*n*=6)	47.3±3.5 **100%** (*n*=6)	8.8±1.2 **100%** (*n*=3)	37.4±1.5 **100%** (*n*=4)
RCAS-Sfrp1	49.2±2 **137.7%***** (*n*=7)	63.1±3.7 **133.4%*** (*n*=6)	6.1±0.5 **68.8%*** (*n*=4)	36.7±1.6 **102%** (*n*=4)

(B) Percentage of cell types generated in the E6 retinas infected with RCAS-dnGSK3β viruses

E6 central retina	Ganglion cells (islet1/2$^+$)	Cone photor (visinin$^+$)	Amacrine cells (CRABP1$^+$)	Mitotic cells (P-H3$^+$)
Control	54.5±2.6 **100%** (*n*=5)	20.2±1.9 **100%** (*n*=3)	14.4±0.8 **100%** (*n*=4)	44.3±5.2 **100%** (*n*=3)
RCAS-dnGSK3β	72.8±3.2 **133.5%***** (*n*=9)	29±1.3 **143.3%** *** (*n*=5)	11.8±0.7 **82.2%*** (*n*=4)	40.7±3.4 **92%** (*n*=3)

Ganglion cells (islet1/2), cone photoreceptors (visinin), amacrine cells (CRABP1) and mitotic cells (P-H3) were counted in Gag-positive areas of the retina and in equivalent regions of the contralateral eye. Only patches falling in the central retina were counted in each animal, although the precise position of the patches varied from embryos to embryos. In each patch, the number of immuno-positive cells was counted in five adjacent sections in a microscope field of 300 μm of length. The data are presented as mean+s.e.m. (Student's *t*-test analysis, ***P<0.005, *P<0.05). Overexpression of Sfrp1 and dnGSK3β (K85R) significantly increased the number of islet1/2- and visinin-positive cells in the retina, whereas the number of CRABP1-positive cells decreased. Cell proliferation was not affected by either Sfrp1 or dnGSK3β overexpression compared with cell proliferation in equivalent zones of the contralateral eye. (*n*), number of embryos analysed.

that in the presence of Wnts GSK3β is inactivated. Interestingly, however, inhibition of GSK3β activity has been also associated with an increase of neuron differentiation in both *Drosophila* (Bourouis et al., 1989) and *Xenopus* embryos (Marcus et al., 1998; Moore et al., 2002). Therefore, we have asked whether SFRP1 activity could involve inhibition of GSK3β, hypothesising that Sfrp1 mechanism of action might be different from that of an extracellular Wnt antagonist.

To this end, we used RCAS-mediated ectopic expression of the dominant-negative form of the *Xenopus* GSK3β [dnGSK3β (Dominguez et al., 1995)], with an experimental design similar to that described for SFRP1 overexpression. In these experiments we observed a retina phenotype that closely matched that of SFRP1 gain-of-function. Statistical analysis showed that, without apparent modifications of the mitotic rate, RGC and cone photoreceptors increased whereas amacrine cells decreased (Table 1B), with percentages that were remarkably similar to those obtained with SFRP1 overexpression (compare Table 1A with 1B). Furthermore, in retina cells, transfection of the dnGSK3β was unable to increase endogenous β-catenin-dependent transcription (Fig. 6B), although this is not surprising because similar results have been reported by others in different cell types (Ding et al., 2000; Smalley et al., 1999).

Besides WNTs, GSK3β is a key component of several signalling pathways. Inhibition of this constitutive active enzyme involves either formation of protein complexes, as in response to WNTs, or phophorylation of specific Ser residues (Cohen and Frame, 2001). Time-course immunoblot analysis with GSK3β phosphorylation specific antibodies demonstrated that SFRP1 CM treatment is followed by a progressive increase in GSK3β phosphorylation at Ser^9, indicative of its inhibition, whereas its phophorylation at Tyr216, which is a measure of the active state (Hughes et al., 1993), did not change between control and treated cultures (Fig. 7A).

A possible explanation of this result was that a trophic factor co-purified with Sfrp1 and produced by MDCK cell line in response to *Sfrp1* transfection was responsible for GSK3β Ser9 phosphorylation. To exclude this possibility, E5 retina explants were electroporated either with *Gfp* or with *Sfrp1* and *Gfp* as a tracer. Explants were grown for 13 hours in defined medium and collected for western blot analysis approximately five hours after protein expression was visually assessed. Without altering the total level of the protein, *Sfrp1* overexpression induced a clear increase in GSK3β Ser9-phosphorylation (Fig. 7B), demonstrating that this modification is likely to be a consequence of *Sfrp1* activity.

Together these results suggest that GSK3β might be an effector of SFRP1 activity in the chick retina.

Discussion

We have shown here with different experimental paradigms that SFRP1, expressed throughout retina development, modulates neuron differentiation, a novel function for this class of molecules. The structural homology between SFRPs and Fz has suggested that, mechanistically, SFRPs could act as competitive antagonists of β-catenin-dependent Wnt signalling, binding and preventing WNT interaction with the Fz receptors (Bafico et al., 1999). Our data provide an additional view showing that SFRP1 activity in the chick retina does not involve the modulation of β-catenin-dependent transcription.

SFRPs have been implicated, as Wnt signalling antagonists, in the control of axis formation in *Xenopus* embryos (Kiecker and Niehrs, 2001), vertebrate somite development (Borello et al., 1999; Lee et al., 2000a), regulation of apoptosis (Ellies et al., 2000; Melkonyan et al., 1997), and kidney and heart formation (Lescher et al., 1998; Schneider and Mercola, 2001; Yoshino et al., 2001). On the basis of the expression pattern in the embryonic chick eye, we have addressed here the role of SFRP1 during retina development. Both gain- and loss-of-function experiments suggested that SFRP1 favoured the number of differentiated neurons in dispersed retina cell cultures. RCAS-mediated overexpression of SFRP1 in ovo extended these observations, demonstrating that SFRP1 favours the generation of both RGC and cone photoreceptors while decreasing the number of amacrine cells. These changes did not imply variations in the rate of mitotic cell division or cell death.

A proposed model for the mechanisms that control retina neurogenesis suggests that the withdrawal of a retina progenitor cell from the cell cycle and its commitment to a differentiated neuron or glia phenotype requires the

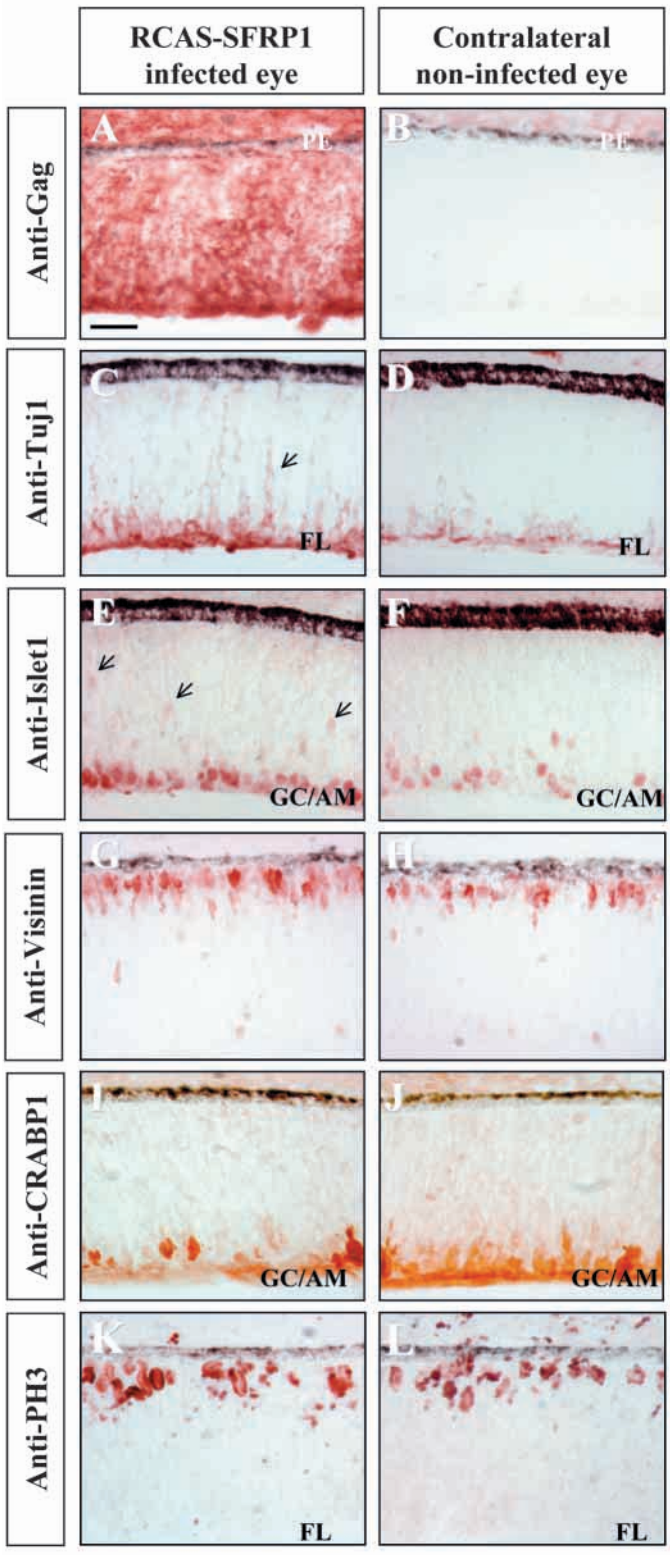

Fig. 5. Overexpression of SFRP1 in chick retinal progenitors increases the number of RGC and cone photoreceptors while decreases the number of amacrine cells. Frontal cryostat sections from retinas of E6 embryos infected with RCAS-SFRP1 viruses at stage 10. Adjacent sections from (A,C,E,G,I,K) infected and (B,D,F,H,J,L) contralateral uninfected central retinas were immunostained with antibodies against the (A,B) Gag viral protein, (C,D) Tuj1, (K,L) the mitotic marker PH3 and the cell-specific differentiation markers (E,F) islet1 (retinal ganglion cells), (G,H) visinin (cone photoreceptors) and (I,J) CRABP1 (amacrine cells). Note how the number of (C,E) cones and migrating (arrowheads) and (A) layered ganglion cells is increased in Gag-positive retinas, while amacrine cells are reduced, without apparent variations in the number of PH3-positive cells. Note also the thickness of the Tuj1-positive fibre layer in (C) the infected retina compared with the thickness of the fibre layer in (D) controls. GC/AM, ganglion cell/amacrine cells; FL, fibre layer; PE, pigment epithelium. Bar, 38 μm.

coordination between a competence state of the precursor cell and its capability of interpreting different extrinsic signals provided by adjacent cells (Livesey and Cepko, 2001). In this context, our findings are consistent with the idea that SFRP1 acts as a differentiation signal on a continuously available pool of progenitor cells. Increased amount of SFRP1 during the first wave of neuron generation appears to drive more progenitor cells into the earliest born retinal cell types, RGC and cones. As a consequence, only a decreased pool of progenitors is available for the generation of neurons born in the second wave of retina neurogenesis. In our analysis we have consistently found a decrease in the number of amacrine cells, which birth date partially overlaps with that of RGC and cones, and, at later stages, a slight decrease in that of horizontal cells. Because these losses appear to be insufficient to compensate for the increase in cones and RGC, it is possible that other cell types might be affected later on, although we were unable to detect it.

Both extrinsic and intrinsic cues contribute to the determination of retina cell fates. Similarly to SFRP1, alterations in the proportion of retina cell types have been observed after manipulations in a number of cues thought to act on already competent progenitors or on post-mitotic cells ‘en route’ to differentiation (Cepko, 1999). These include transcription factors of the bHLH family, such as the *Ath5* or *NeuroD* genes (Brown et al., 2001; Kay et al., 2001; Moore et al., 2002; Morrow et al., 1999; Wang et al., 2001), or secreted signalling molecules such as CNTF or FGFs (Ezzeddine et al., 1997; McFarlane et al., 1998; Patel and McFarlane, 2000). Our experimental design (overexpression during early neurogenesis) does not allow us to distinguish whether SFRP1 is a specific selector for RGC and cone photoreceptors or whether it just pushes more cells toward differentiation into the scheduled phenotype. The dynamic expression of *Sfrp1*, which follows the wave of neurogenesis in the retina, might support the latter hypothesis. Should this be the case, SFRP1 overexpression at later stages of neurogenesis would increase, for instance, the number of amacrine/horizontal cells over bipolar cells.

The activation of the Delta-Notch signalling pathway is one of the key events in the process of cell differentiation. Although Notch signalling may be required at different stages of retina development (Livesey and Cepko, 2001), interference with its activity at early stages of development in the chick increased the production of RGC (Austin et al., 1995; Henrique et al., 1997). Because Notch activation decreases *Delta* expression, it has been proposed that waves of *Delta* expression may control the numbers of retina progenitors competent to respond to time-controlled inductive signals, thus allowing the generation

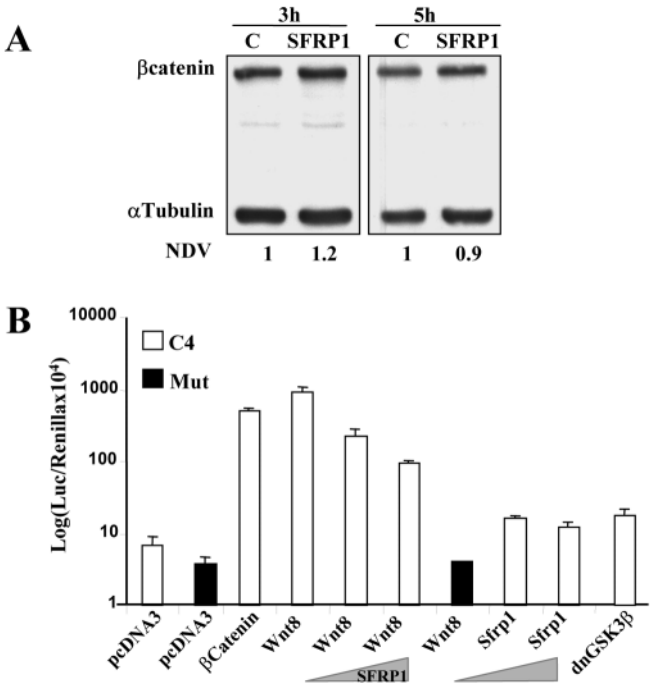

Fig. 6. SFRP1 activities do not modify β-catenin transcriptional activity. (A) Immunoblot analysis of cytosolic β-catenin levels in MDCK/control or MDCK/SFRP1 treated retina cells harvested after three and five hours of culture. Cytosolic fractions were prepared and immunoblotted with antibodies against β-catenin and α-tubulin (load control). Normalized density values (NDV) expressed in arbitrary units indicate no differences among the fractions. (B) Luciferase assay showing that SFRP1 does not modulate the endogenous level of β-catenin/TCF-dependent transcription in E5 chick dissociated retinal cells. Cells were co-transfected with the reporter plasmid containing the Lef-1 responsive element (C4) or its mutated version (MUT), the control plasmid pRLTK and the effector plasmids in each case. Either 500 ng or 1 μg of *Sfrp1* were transfected alone or in combination with 500 ng of *Wnt8*. 500 ng of *Wnt8* or β-*catenin* activate the reporter gene 240 and 130 fold, respectively. The two concentrations of *Sfrp1* decreased *Wnt8* activity 4 and 10 fold, respectively. 1 μg of dnGSK3β did not activate the reporter. Luciferase activity is expressed in a logaritmic scale.

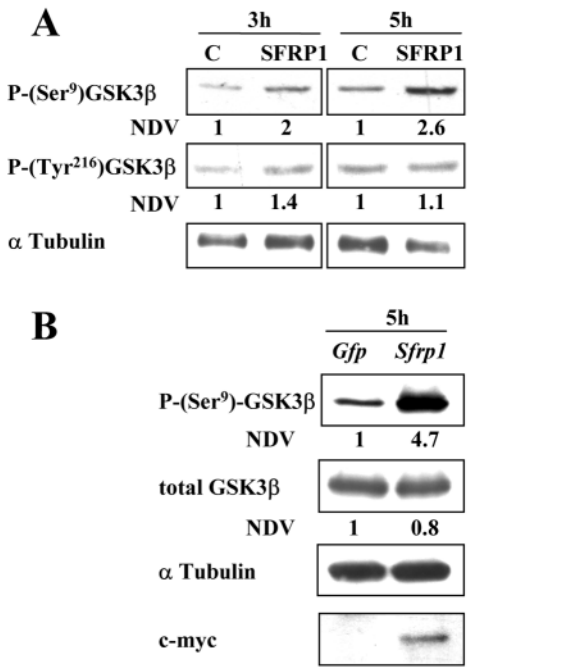

Fig. 7. Treatment of retina cells with SFRP1 is associated with phospho-Ser^9-mediated inhibition of GSK3β activity. (A) SFRP1-inhibits GSK3β activity by phosphorylation at Ser^9. SFRP1- or control-treated retina cells, harvested three or five hours after the culture, were analysed by immunoblots with antibodies α-GSK3β-phospho-Ser^9 (inactive form), GSK3β-phospho-Tyr^{216} (active form) and α-tubulin, as loading control. NDV indicate a clear increase in phospho-Ser^9-mediated inhibition of GSK3β activity. (B) The total and phospho-Ser^9-fraction of GSK3β was evaluated with specific antibodies by western blots in retina explants electroporated with either Gfp or Sfrp1-myc and collected five hours after protein expression. Total content of GSK3β was normalised against α-tubulin. Phospho-Ser^9 positive bands were normalised against total GSK3β content. Anti-myc demonstrates Sfrp1-myc expression after electroporation.

of sequentially ordered cell fates (Dorsky et al., 1997). SFRP1 overexpression did not appear to modify the level of *Delta* expression in the retina, and ectopic expression of SFRP1 in the proliferating peripheral retina did not initiate ectopic neuron production. Altogether these data indicate that only already competent cells could differentiate upon exposure to SFRP1.

Wnt genes were first described in the CNS (Wilkinson et al., 1987); nevertheless, their functions in this tissue are still poorly understood. Different WNTs have been implicated in the control of neural progenitor proliferation (Dickinson et al., 1994; Ikeya et al., 1997; Lee et al., 2000b). Recently, it has been proposed that in the neural tube this function might be achieved through the establishment of a mitogenic gradient, that, upon activation of the canonical signalling pathway exerts a transcriptional regulation of cell cycle specific genes, controlling the final size and shape of the neural tube across the dorso-ventral axis (Megason and McMahon, 2002). On the basis of these observations and on the proposed function for SFRP proteins, SFRP1-mediated effects observed in the retina could be explained as the results of SFRP1 interference with endogenously present mitogenic, β-catenin-dependent WNTs. Our results, however, do not easily support this possibility. Comparison of the reporter activity of a wild-type or a mutated version of the TCF-responsive element suggests that E5 chick retina cells have a very low basal β-catenin transcriptional activity. This activity was not modified by different amounts of *Sfrp1*, although *Sfrp1* could, at least in part, repress the activation of β-catenin transcriptional activity induced by *Wnt8* transfection. These data suggest that retina cells are competent to respond to Wnt signalling and that SFRP1 has the potential of antagonising WNTs, when these are exogenously added. However, SFRP1 activity in the retina appears independent at least of those WNTs that activate the canonical pathway. Theses results are in agreement with a recent study in zebrafish embryos that shows that, in the eye, cells from the pigment epithelium, lens and ciliary margin but not those from the neural retina are potential β-catenin-responsive cells (Dorsky et al., 2002).

Besides β-catenin, Wnt signalling can lead either to the activation of protein kinase C or to Jun-kinase mediated

transcription. At the moment we cannot exclude that SFRP1 activity in the retina interferes with one of these pathway. However, initial experiment aimed at determining this possibility, do not support this idea (data not shown). Instead our results indicate that SFRP1 treatment is followed by a phospho-Ser^9 mediated inhibition of GSK3β, and RCAS-mediated ectopic expression of a dominant-negative form of GSK3β induced a retina phenotype remarkably similar to that obtained with SFRP1 gain-of-function. Phosphorylation mediated inhibition of GSK3β is a central step over which the signalling cascades of several different trophic factors converge, but this is not the mechanism implied in GSK3β inhibition in the canonical Wnt pathway (Ding et al., 2000). Therefore our results are, again, not in line with the proposed function for SFRPs. Our results do not establish a direct link between SFRP1 and GSK3β phosphorylation and we cannot totally exclude that RCAS-SFRP1 and RCAS-dnGSK3β mediated phenotypes in the retina might be a mere coincidence. However, frizzled signalling has been proposed as a possible trigger for similar GSK3β-mediated phenotypes observed in the *Xenopus* retina, where GSK3β activity seems required for the post-translational control of different bHLH molecules, needed for the time-controlled generation of the various retina neurons (Moore et al., 2002). Whether SFRP1 might be part of this trigger is just a matter of speculation. As a hypothesis, in absence of WNTs molecule, SFRP1 could bind directly to a Fz receptor through interaction of their respective CRD domains. Indeed, SFRP1 can form complexes with a Fz prototype (Bafico et al., 1999), and dimerization and multimerization of the CRD domains in both Fz and SFRPs appears as a necessary step for their function (Dann et al., 2001). Fz activation or SFRP1 binding to a different receptor (i.e. a receptor with intrinsic protein tyrosine kinase activity) could lead to activation of a kinase that, in turn, would be responsible for the phospho-Ser9-mediated inactivation of GSK3β (Cohen and Frame, 2001), followed by the post-transcriptional control of genes, as those related to atonal, required for the acquisition of specific cell phenotype (Moore et al., 2002). This model does not exclude that, in other tissues, in the presence of WNT, SFRP1 may interfere with the canonical signalling pathway as an extra-cellular antagonist, as previously described (Bafico et al., 1999; Xu et al., 1998).

In conclusion, we propose that SFRP1 contributes to retina development with a mechanism that does not involve preventing WNT-mediated activation of its canonical signalling pathway. Whether SFRP1 interferes with non-canonical Wnt signalling still needs to be determined.

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