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Negative Regulation of Lens Fiber Cell Differentiation by RTK Antagonists Spry and Spred

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Abstract

Sprouty (Spry) and Spred proteins have been identified as closely related negative regulators of the receptor tyrosine kinase (RTK)-mediated MAPK pathway, inhibiting cellular proliferation, migration and differentiation in many systems. As the different members of this antagonist family are strongly expressed in the lens epithelium in overlapping patterns, in this study we used lens epithelial explants to examine the impact of these different antagonists on the morphologic and molecular changes associated with fibroblast growth factor (FGF)-induced lens fiber differentiation. Cells in lens epithelial explants were transfected using different approaches to overexpress the different Spry (Spry1, Spry2) and Spred (Spred1, Spred2, Spred3) members, and we compared their ability to undergo FGF-induced fiber differentiation. In cells overexpressing any of the antagonists, the propensity for FGF-induced cell elongation was significantly reduced, indicative of a block to lens fiber differentiation. Of these antagonists, Spry1 and Spred2 appeared to be the most potent among their respective family members, demonstrating the greatest block in FGF-induced fiber differentiation based on the percentage of cells that failed to elongate. Consistent with the reported activity of Spry and Spred, we show that overexpression of Spry2 was able to suppress FGF-induced ERK1/2 phosphorylation in lens cells, as well as the ERK1/2-dependent fiber-specific marker Prox1, but not the accumulation of β-crystallins. Taken together, Spry and Spred proteins that are predominantly expressed in the lens epithelium in situ, appear to have overlapping effects on negatively regulating ERK1/2-signaling associated with FGF-induced lens epithelial cell elongation leading to fiber differentiation. This highlights the important regulatory role for these RTK antagonists in establishing and maintaining the distinct architecture and polarity of the lens.

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Keywords

Lens fiber differentiation; FGF; RTK-antagonists; Sprouty; Spred

1. Introduction

The establishment and maintenance of the distinctive architecture and polarity of the ocular lens is dependent on the regulated proliferation of its anterior epithelia, and the subsequent differentiation of these cells into fibers that constitute the bulk of the lens (Lovicu and McAvoy, 2005). These lens cellular processes are induced by many different ocular-derived growth factors, with fibroblast growth factor (FGF) a key inducer of lens fiber differentiation (Chamberlain and McAvoy, 1989; Lovicu and McAvoy, 2005; Zhao et al., 2008). FGF-induced lens fiber differentiation is dependent on the synchronized and integrated action of different intracellular signaling pathways (Chamberlain and McAvoy, 1989; Lang and McAvoy, 2004; Lovicu and McAvoy, 2005), with receptor tyrosine kinase (RTK)-induced Ras-ERK/MAPK-signaling playing an integral role (Iyengar et al., 2007; Upadhya et al., 2013). Levels of ERK1/2-signaling have been shown to be spatially and temporally regulated in lens cells to elicit a fiber differentiation response (Iyengar et al., 2007), and this regulation is proposed to be at least partly achieved by inhibitory signals provided by RTK-antagonists, namely members of the Sef, Sprouty (Spry) and Spred (Sprouty-related Ena/VASP homology-1-domain-containing proteins) families (see Shin et al., 2015).

Spry was one of the first bona fide feedback regulators of the FGF pathway, initially identified in *Drosophila* (Hacohen et al., 1998; Tefft et al., 1999). The four mammalian Spry isoforms are approximately 32–34 kDa, and differ at their N-terminus (Mason et al., 2006; Matsumura et al., 2011), conferring their ability to interact with other proteins, dictating their putative differential function (Kim and Bar-Sagi, 2004). All mammalian Spry proteins share a conserved cysteine-rich domain at their carboxyl terminus, as well as another short region containing a conserved tyrosine residue (Tyr55/*Spry2*, Tyr53/*Spry1* & *Spry4*) that functions as a binding site for the SH2 domain of Grb2 (Hanafusa et al., 2002; Fong et al., 2003; Hall et al., 2003; Kim and Bar-Sagi, 2004). In the case of FGF-signaling, phosphorylated Tyr55 of Spry2 associates with Grb2, blocking the interaction of Grb2 with the FGF receptor adaptor molecule, FRS2, that bridges the receptor to the ERK/MAPK pathway (Kovalenko et al., 2003). This is in contrast to Sef that acts primarily at the level of the FGF receptor, directly blocking the phosphorylation of FRS2 (Kavalenko et al., 2006).

Spreds are evolutionary-conserved membrane-associated negative regulators of growth factor-induced ERK/MAPK activation, related to Sprouty (Hanafusa et al., 2002; Kavalenko et al., 2006; Bundschu et al., 2007). The three mammalian Spred genes (*Spred-1, Spred-2 and Spred-3*) consist of 6–7 exons and encode proteins of approximately 400–450 a.a. The proteins primarily consist of three domains; a 100 a.a. N-terminal protein interaction EVH1 domain (Enabled/VASP homology 1) that binds proline-rich sequences (Ball et al., 2002), targeting Spreds to specific cellular sites; a central 50 a.a. KBD domain (c-Kit binding domain, absent in Spred-3) that allows tyrosine kinase interaction (Engelhardt et al., 2004; Bundschu et al., 2007); and a C-terminal SPR domain (Sprouty-related domain), similar to

the conserved cysteine-rich C-terminus of Sprouty proteins, required for membrane anchoring, Spred heterodimer formation, and interaction with c-Kit to suppress ERK phosphorylation (Wakioka et al., 2001; Bundschu et al., 2007). Although the EVHI domain is essential for Spred protein function in inhibiting ERK activation, its reduced size in Spred-2 still permits inhibition of cell differentiation (Bundschu et al., 2007), suggesting Spred-2 may have different mechanisms for regulation of different cellular activities.

Many different studies have reported on the inhibitory effects of Sef, Spry and Spred on biological processes induced by different growth factors (Mason et al., 2006; Bundschu et al., 2007; Cabrita and Christofori, 2008; Ron et al., 2008), with a multitude of signaling pathways shown to be regulated by Spry and Spred proteins (Neben et al., 2017). More specifically, many of these reports have shown these antagonists to negatively regulate FGF activity, in particular the Ras-ERK/MAPK pathway (Hacohen et al., 1998; Kramer et al., 1999; Casci et al., 1999; Chambers and Mason, 2000; Wakioka et al., 2001; Kim and Bar-Sagi, 2004). Sef, Spry and Spred proteins are expressed in various tissues and organs, including lens and eye throughout embryogenesis and postnatal growth (Boros et al., 2006; Zhao et al., 2015). Interestingly, Spry expression often localizes to the same sites of FGF-signaling during murine embryogenesis (Chambers and Mason, 2000), potentially antagonizing Ras-Raf-ERK1/2-signaling downstream of the FGF receptor (FGFR; Minowada et al., 1999; Chambers and Mason, 2000; Hanafusa et al., 2002). Normal expression of Sef, Spry1 and Spry2 has been shown in lens epithelial cells and primary fiber cells, with transcripts progressively lost in maturing primary fiber cells with fetal and postnatal growth (Boros et al., 2006). Spred1, Spred2 and Spred3 are also expressed in ocular tissues, including lens, with higher expression levels of Spred family proteins in the lens epithelium, compared to the mature lens fiber cells (Zhao et al., 2015). These distinct temporal and spatial patterns of expression in lens not only correlate with the morphologic changes of lens epithelial cells to fiber cells at the lens equator, but also with increased activity (phosphorylation) of ERK1/2 at this site.

In vivo studies using transgenic mice have provided some insights into the efficacy of these antagonists, with their mis-expression disrupting lens morphogenesis and/or fiber differentiation. As mentioned, Sef is known to specifically inhibit FGFR-signaling by either directly antagonizing the FGFR (Tsang et al., 2002) and/or by blocking elements of the FGFR-activated ERK1/2-pathway (Torii et al., 2004). Overexpression of Sef in lens of transgenic mice resulted in a smaller lens phenotype, due to direct inhibition of cell elongation associated with FGF-induced primary and secondary fiber differentiation (Newitt et al., 2010). Taken together with the fact that relatively lower levels of FGF-activity are important for maintenance of the proliferative lens epithelium (McAvoy and Chamberlain, 1989), these findings are strongly suggestive that Sef may normally play a role as a specific negative-regulator of FGF-activity in the lens epithelium (Newitt et al., 2010). More recent studies have also overexpressed Spry in lens (Shin et al., 2015), and while this resulted in a similar embryonic phenotype of a small lens as seen with Sef, fiber cell differentiation was compromised but not in the exact same manner as for Sef transgenic mice. Further *in vitro* studies, using lens epithelial explants from the Spry gain of function mice, showed that FGF-induced fiber differentiation was compromised, with impaired cell elongation (Shin et al., 2015), similar to the actions of Sef.

Given Sef, Spry and Spreds have all been shown to be expressed in similar and overlapping patterns in the lens, and that they appear to antagonise similar downstream signaling pathways (Wakioka et al., 2001), there is clearly potential overlap in their functional roles in lens, especially in relation to the regulation of lens fiber differentiation. This is highlighted by the fact that Sef-deficient mice do not present a lens phenotype (Newitt et al., 2010). To better understand the role of the different Spry and Spred antagonists as regulators of FGF-induced RTK-signaling in lens leading to fiber differentiation, we used different approaches to overexpress these different molecules in epithelial cells of rat lens explants, primarily to compare the efficacy of the different inhibitors on FGF-induced lens fiber differentiation. Here we demonstrate for the first time the functionally overlapping effects of the Spry and Spred members in lens, in that increased expression of either Spry1, Spry2, Spred1, Spred2 or Spred3 in lens epithelial cells is sufficient to suppress FGF-induced cell elongation leading to fiber differentiation, with Spry1 and Spred2 being the most effective in our transfection studies. This inhibition mediated by these antagonists appears to act via suppressing the levels of ERK1/2 phosphorylation, once again highlighting the significant role of this signaling pathway in orchestrating aspects of the fiber differentiation process, in particular the integral elongation of these cells.

2. Materials and Methods

All animal handling and operating procedures carried out in this study adhered to the ARVO statement for the use of animals in ophthalmic research, conforming to the provisions of the code of practice provided by the National Health and Medical Research Council (NHMRC, Australia), and approved by the Animal Ethics Committee of the University of Sydney, NSW, Australia.

2.1. Preparation of lens epithelial explants

All ocular tissues were derived from postnatal-day-10 (P10) albino Wistar rats (*rattus norvegicus*). Lens epithelial explants were prepared as described previously (Lovicu and McAvoy, 2008), and were cultured in Medium 199 with Earle's salts (M199; Trace Scientific, NSW, Australia), supplemented with 0.1% bovine serum albumin (BSA; Sigma, NSW, Australia), 0.1 μg/ml L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml Amphostat (all from Trace Scientific), at 37°C in 5% CO_2. Human recombinant FGF2 (50–100 ng/ml; R&D Systems; MN, USA) applied to lens explants was prepared as per manufacturer's instructions.

2.2. Heterologous gene expression in lens epithelial explants

Genes of interest were expressed in lens epithelial explants using either a lipid-based approach (Lipofectamine® LTX) or by adenoviral transduction.

2.2.1. Lipofectamine® LTX transfection—Full length murine cDNA coding for RTK antagonists from the Spry and Spred families were cloned into the pLXSG reporter plasmid, a retroviral expression vector derived from the MMLV-based recombinant vector pLXSN (Clontech, CA, USA; see Miller and Rosman, 1989), whereby the neomycin resistance gene of pLXSN is replaced by cDNA encoding enhanced green fluorescence protein (EGFP). The

following vectors were generated: pLXSG-Spry1, pLXSG-Spry2, pLXSG-Y55A-Spry2 (a non-functional Spry2 containing a Y55A missense mutation; see Mason et al., 2004), pLXSG-Spred1, pLXSG-Spred2 and pLXSG-Spred3. Y55A-Spry2 was adopted as the main control gene, as missense mutants of the other Spry and Spred family members were not readily available. The plasmids were purified according to manufacturer's instructions (Astral Scientific, Australia) and all inserted gene sequences were confirmed as previously described (Zhao et al., 2015).

Transfection of lens epithelial explants using Lipofectamine® LTX was based on previous studies (Madakashira et al., 2012; Zhao et al., 2015; Shin et al., 2015). In brief, 2μg of plasmid DNA coding for the antagonist of interest, co-expressing EGFP, was diluted in 45μl of M199 without antibiotics. PLUS Reagent (Invitrogen, CA, USA) was added to this diluted DNA (1:10), mixed gently and incubated for 10 minutes at room temperature. Five microliters of Lipofectamine® LTX Reagent (Invitrogen, USA) was also diluted 1:10 in M199 media without antibiotics and mixed gently, before combining both mixtures and incubating them at room temperature for 30 minutes to form the DNA-lipid complexes. Prior to cell transfection, lens epithelial explants were rinsed with M199 without antibiotics (3 × 5 minutes), and fresh M199 without antibiotics (900μl), supplemented with the 100μl of the DNA-lipid complexes, was added to each dish. The explants were incubated at 37°C in 5% CO_2 for 48 hrs. The epithelial cells were visualized using epi-fluorescent microscopy (Olympus CKX41, Tokyo, Japan) to confirm successful transfection (expression of EGFP). The media was then replaced with 1ml of fresh M199 supplemented with antibiotics, and the explants were subsequently treated with a differentiating dose of FGF2 and monitored and photographed (Leica Firecam, Germany) for up to 5 days.

2.2.2. Adenoviral transduction—Human adenovirus serotype 5 (Ad5)-based vectors were alternatively used to transduce genes of interest in primary lens epithelial cells. The recombinant adenoviruses were generated using the AdEasy system (He et al., 1998). The RTK antagonist cDNAs were cloned into a shuttle vector (pAdTrack-CMV) that incorporates the EGFP reporter gene (to trace all steps involved in virus production). Each gene was PCR ampflied from the parent pLXSG vector with PCR primers containing unique restriction sites (5′*NotI* and 3′*EcoRV* or 3′*AfeI*) and cloned into the *NotI/EcoRV* site of pAdTrackCMV. The resultant construct was linearized with *PmeI* and co-transformed with a supercoiled adenoviral vector (e.g. pAdEasy-1) into *rec*$^+$ *E. Coli* (BJ5183 cells). Recombinants were selected for kanamycin resistance, further screened by multiple restriction endonuclease digestion, and linearized with *PacI* to expose the inverted terminal repeats for transfection into HEK293T packaging cells. The adenoviral DNA was transfected using calcium phosphate precipitation and upon the appearance of cytopathic effects after 7 to 10 days, the cells and supernatant were harvested at 2,500 rpm in 50 mL Falcon tubes. Each cell pellet was resuspended in 1.0 mL 10 mM Tris-Cl, pH7.6 and then subjected to 3 cycles of freeze/thawing to lyse the cells. The clarified lysate was collected after centrifugation and was amplified by infecting more packaging cells. After successful amplification, clarified lysates were loaded onto $CsCl_2$ density gradients and spun at 20,000 rpm for a minimum of 4 hr in an SW28 swing bucket rotor in a Beckman ultracentrifuge. The crude adenoviral band was extracted via syringe and 16-gauge needle and then

subjected to additional ultracentrifugation in an SW41 rotor spun at 40,000 rpm for 16 hr. A band corresponding to fully encapsidated adenoviral particles was extracted by syringe and then dialysed in a Slide-a-lyzer cassette (10,000 MWCO, Thermo Scientific) in 1 L of 3% (w/v) sucrose in PBS for 1 hr. The buffer was exchanged twice, before the adenoviral particles were removed and then snap frozen in liquid nitrogen and stored at −70°C for subsequent experiments.

Lens epithelial explants were replenished with 900μl of fresh M199 without antibiotics. Adenoviral vectors including control (Ad5EGFP) or carrying the RTK antagonists of interest (Ad5Spry1, Ad5Spry2 and Ad5Spred2), including the mutant Spry2 (Ad5-Y55A-Spry2), were diluted in 100μl of M199 and added to each dish (~10^5 plaque-forming unit, pfu/ml). The explants were incubated at 37°C in 5% CO_2 for 3 to 5 hrs, before substituting the 'infected' media with 1ml fresh media with antibiotics to incubate at 37°C in 5% CO_2 for 48 hrs. The epithelial cells were visualized using phase contrast and inverted epi-fluorescent microscopy (Olympus CKX41) to confirm successful transfection of the explants (expression of EGFP). The media was again replaced with 1ml of fresh M199 supplemented with antibiotics and the explants were subsequently treated with a fiber-differentiating dose of FGF2 (50 ng/ml) for up to 5 days.

2.3. SDS-PAGE and western blotting

Lens explants were collected and homogenized in cold lysis buffer containing 2.5 mM EDTA, 25 mM Tris, 0.375 M NaCl, 1.5 mM $NaVO_3$, 1% IGEPAL, 1% (v/v) Na-deoxycholate, 1%(v/v) Triton-X (Sigma-Aldrich; Australia), 0.1% (v/v) SDS, protease inhibitor cocktail tablet (Complete Mini, Roche Diagnostics, Germany) and Phosstop Easypack phosphatase inhibitor (Roche Diagnostics, Germany). To facilitate lysis, the homogenized samples were rotated for 2hr at 4°C. Following protein quantification using the Micro-BCA protein assay kit (Pierce, IL, USA) 5μg of total protein was run on a 10% SDS-PAGE gel. Prior to gel loading, protein lysates were mixed in a 1:1 ratio with Laemmli sample buffer (BioRad, CA, USA) containing 5% (v/v) 2-mercaptoethanol as a denaturant. Once separated, proteins were transferred from the gel matrix to a PVDF membrane (Millipore; MA, USA). The PVDF membrane was incubated in blocking buffer (5% (w/v) skim milk/TBST or 2.5% (w/v) BSA/TBST for phosphoproteins) for 1 hr at room temperature. They were then incubated with primary antibody diluted in blocking buffer at 4°C overnight. Antibodies against Spred2 (ab153700, Abcam, VIC, Australia), Spry1 and Spry2 (sc-30048, sc-30049; Santa Cruz, USA) were all diluted 1:500, and phosphorylated ERK1/2, total ERK1/2 (9106s, 9102s; Cell Signaling Technologies, USA), Prox1 (courtesy of Prof M. Duncan, Delaware, USA) and β-crystallin were all diluted 1:1000. Anti-GAPDH antibody (Sigma-Aldrich, USA) was used at a 1:5000 dilution. On the following day, the primary antibodies were removed by washing in TBST (3 × 5 minutes), and the membranes were then incubated with the appropriate secondary antibody at room temperature for 2 hrs. For GAPDH, rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Cell Signaling Technology) was used at a 1:5000 dilution. For all the other primary antibodies, goat anti-rabbit HRP-conjugated IgG (Cell Signaling Technology, USA) was used at a 1:5000 dilution. After washing in TBST (3 × 10 mins), the membranes were incubated in Immobilon Western Chemiluminescent HRP substrate (Millipore) for 5 minutes. The signals

were detected with a ChemiDoc™ imaging system (Bio-Rad Laboratories, CA, USA). The intensity of protein bands was analyzed using Image Lab software (Bio-Rad Laboratories), and all levels of proteins of interest were normalized relative to corresponding GAPDH levels.

2.4. Immunofluorescent labeling of explants

Prior to immunofluorescent labeling, explants were viewed and captured under epifluorescence (Olympus CKX41). Explants were then fixed in 10% NBF for 5 minutes, rinsed in PBS/BSA buffer (3 × 5 minutes), permeabilized in PBS/BSA containing 0.05% (v/v) Tween-20 (Sigma-Aldrich; Australia) (3×5 minutes), followed by two 5-minute washes in PBS/BSA. Explants were then blocked in 3% (v/v) normal goat serum (NGS)/PBS for 30 minutes at room temperature, before incubating overnight with the primary antibody of interest at 4°C in a humidified chamber. Antibodies against β-catenin (D0215, Santa Cruz) or β-crystallin were diluted in 3% NGS/PBS, 1:100 or 1:50, respectively.

Explants were brought to room temperature (30 minutes) and were rinsed with PBS/BSA (3 × 5 minutes), followed by incubation in the corresponding secondary antibody, at room temperature, in the dark for 2 hr in a humidified chamber. Anti-rabbit Cy3-conjugated (1:1000; Sigma-Aldrich; Australia) and anti-rabbit Alexa Fluor 488 (1:1000; Sigma-Aldrich) were diluted in PBS/BSA. Explants were then rinsed (2 × 5 minutes each) in PBS/BSA, and cell nuclei counterstained with Hoechst 33342 dye for 3 minutes. Explants were rinsed with PBS/BSA (3 × 5 minutes), and then mounted in 10% (v/v) PBS in glycerol. The cells were imaged under an epi-fluorescent research microscope (Leica-DMLB and Leica DC-180; Germany) or the LSM5 Pascal confocal microscope (Carl Zeiss; USA).

2.5. Quantification of FGF-induced differentiation

The successfully transfected lens epithelial cells expressing EGFP were photographed after 5 days of culture. All cell counts were carried out by a researcher blinded to the sample identity. An epithelial cell was defined as being cobblestone in shape, similar to epithelial cells prior to the addition of any factors. Any cell that was elongated in appearance, increasing in length at least three times that of a control epithelial cell was considered to have undergone elongation and hence fiber differentiation. The percentage of labeled cells undergoing elongation/fiber differentiation was determined and statistical significance was inferred using an unpaired two tailed t-test, with a p-value less than 0.05 considered statistically significant.

3. Results

3.1. FGF-induced lens fiber differentiation

Lens epithelial explants treated with or without FGF were collected after 5 days of culture. After 5 days of treatment with no FGF (control), lens epithelial explants showed no notable changes in cell shape over the culture period, with the monolayer of epithelial cells retaining their cobblestone morphology over the 5 days (Fig. S1A). In contrast, explants treated with FGF2 showed uniform labeling for β-crystallin, with prominent cell elongation and multilayering (Fig. S1B).

3.2. Lipofectamine® transfection

3.2.1. RTK antagonists block FGF-induced lens cell elongation—To examine the efficacy of the different RTK inhibitors in preventing FGF-induced fiber differentiation, cells in lens epithelial explants were transfected with a panel of different antagonists normally expressed in the lens (i.e. Spry1, Spry2, Spred1, Spred2 and Spred3) including a mutant, non-functional Spry2 (Y55A-Spry2, control), and the reporter plasmid (pLXSG, control). Successful transfection was evident by the expression of reporter protein (EGFP), indicative of plasmid uptake by the cells, that allowed us to visualise individual cells (Fig. 1 and Fig. 2). Using β-catenin to label cell membranes, we could observe the EGFP-positive transfected cells in the context of the surrounding non-transfected cells within the explant (Fig. 1 and Fig. 2).

When explants were transfected with the control empty vector (pLXSG) alone and cultured without FGF for 5 days (control, Fig. 1A), the EGFP-expressing cells maintained their cuboidal shape and no elongation was evident, indicating that EGFP-expression has no direct influence on lens epithelial cell integrity. When these control-transfected cells were treated with FGF, they underwent cell elongation (Fig. 1B, B′), along with their neighbouring non-transfected cells, typical of the FGF-induced fiber differentiation response. β-catenin labeling of the cell membranes highlighted the elongation of both transfected and non-transfected cells (Fig. 2B). The transfection process did not influence the ability of cells to normally respond to FGF.

When rat lens epithelial explants were transfected with the antagonists of interest to overexpress either Spry1 or Spry2, they maintained their cuboidal shape (Fig. 1C and 1E) over 5-days culture without FGF. The non-transfected cells surrounding the transfected cells did not show any morphological changes without FGF, as indicated by β-catenin membrane labeling (Fig. 1C and 1E). In the presence of FGF (Fig. 1D and 1F), many of the transfected cells overexpressing Spry1 or Spry2 had retained their normal morphology and did not elongate. In contrast, β-catenin staining showed that the surrounding non-transfected cells in the presence of FGF lost their epithelial morphology and elongated (Fig. 1D and 1F), acting as internal controls. While this demonstrated that overexpression of specific antagonists in lens epithelial cells has the ability to block FGF-induced cell elongation and differentiation, some EGFP-positive cells still underwent FGF-induced elongation indicating incomplete inhibition in this model. For example, as shown in Fig. 1D′ and 1F′, both normal cuboidal shape epithelial cells and elongated fiber cells (shown by arrows) were observed in the same explants transfected with Spry1 or Spry2, following treatment with FGF. As a positive control group, we overexpressed an inactive phospho-mutant form of Spry2 (Y55A-Spry2) in lens epithelial explants (Fig. 1G and 1H). In the presence of FGF, epithelial cells transfected with pLXSG-Y55A-Spry2 elongated (Fig. 1H, H′), similar to the surrounding non-transfected cells labeled for β-catenin (Fig. 1H), and FGF-treated lens epithelial cells in control explants (Fig. 1B).

Similar to Spry proteins, members of the Spred protein family could also inhibit FGF-induced lens fiber differentiation. In the absence of FGF, cells in lens epithelial explants transfected with Spred1, Spred2 or Spred3 (Fig. 2A, 2C and 2E) retained their normal

cellular morphology after 5 days of culture. In the presence of FGF, most of the cells overexpressing Spred1, Spred2 or Spred3 (Fig. 2B, 2D and 2F) maintained this cuboidal shape. Again, β-catenin labeling of cell membranes showed that the surrounding non-transfected cells could still respond to FGF, losing their epithelial morphology and elongating (Fig. 2B, 2D and 2F). While this indicated that overexpression of Spreds prevents lens epithelial cells from elongating in response to FGF, like for Spry overexpression, a small proportion of the transfected cells expressing Spreds were still able to elongate with FGF treatment after 5 days (as shown by arrows in Fig. 2B′, 2D′ and 2F′).

3.2.2. Cell counts and statistical analysis—Approximately 64% of the control-transfected cells were elongated by 5 days culture in the presence of FGF (Fig. 3), consistent with previous studies (Shin et al., 2015). We saw a significant reduction in the elongation of Spry1- or Spry2-expressing cells treated with FGF, compared to the control-transfected explants (Fig. 3; student's *t*-test; $p<0.005$). Expression of Spry2 mutant (Y55A-Spry2) abrogated the block of FGF-indued fiber cell elongation observed with Spry2 (58% cells elongated, Fig. 3, student's *t*-test; $p<0.005$), similar to control levels (Fig. 3, student's *t*-test; $p>0.3$). Similarly to Spry proteins, lens explants with cells overexpressing Spred1, Spred2 or Spred3 also showed a significant decrease in the percentage of FGF-induced cell elongation, reduced to 37%, 24% and 35% respectively (Fig. 3; $p<0.001$). Spred2 appeared to be the most potent inhibitor of FGF-induced cellular changes (67% reduction in number of transfected cells that elongated compared to controls, student's *t*-test; $p<0.05$), with similar reduced percentages in FGF-induced cell elongation (student's *t*-test; $p>0.1$) shown for Spred1-expressing cells (43% reduction in number of transfected cells that elongated compared to controls) and Spred3-expressing cells (46% reduction in number of transfected cells that elongated compared to controls).

3.3. Adenoviral transduction studies

3.3.1. Increased transfection efficiency by using adenoviral transduction system—Our transfection studies demonstrated some evidence of RTK-antagonists inhibiting FGF-induced lens fiber differentiation; however, the relatively low transfection efficiency did not permit a more detailed molecular analyses of the cellular changes. To increase gene transfer efficiency in primary lens epithelial cells in explants, we used adenoviral vectors. Using a similar reporter gene co-expression approach described earlier, we were able to obtain a significantly higher EGFP expression in lens epithelial explants using adenovirus (see Fig. S2B), compared to liposome-mediated transfection (see Fig. S2A). We could now reliably characterise protein changes in these cells in the presence and absence of our antagonists. Based on the transfection study (Fig. 1 and Fig. 2), Spry1 and Spred2 were the most effective antagonists and were used here. For comparison, we also transduced cells with WT and mutant Spry. We show the efficiency of this approach by first demonstrating that the levels of all three antagonist proteins (Spry1, Spry2 and Spred2) are markedly increased in our transduced cells after 48 hrs, compared to endogenous levels in control-transduced cells (Fig. S2C).

3.3.2. Overexpression of Spry suppressed FGF-induced phosphorylation of ERK1/2—Given the MAPK/ERK1/2 pathway is a well-established target of the Spry and

Spred antagonists, we examined the levels of ERK1/2 phosphorylation in non-transduced explants over 10 hr of culture with a differentiating dose (50 ng/ml) of FGF. ERK1/2 phosphorylation increased over time as expected (Fig. 4A). In epithelial cells transduced with Spry2 and similarly treated with FGF, the levels of phosphorylated ERK1/2 were significantly lower over much of the culture period (Fig. 4B), whereas in Y55A-Spry-transduced explants, levels of phosphorylated ERK1/2 increased by 1 hr of culture, remaining elevated over the 10 hr (Fig. 4C), similar to non-transfected cells (Fig. 4A). Quantitative analysis of pERK1/2 and total ERK1/2 levels, normalized to GAPDH levels, confirmed these differences, with significant reductions in ERK1/2 phosphorylation in lens explants overexpressing Spry2 at 2 to 8 hr (Fig. 4D, student's *t*-test; $p<0.05$), highlighting the reduced ability of FGF to induce phosphorylation of ERK1/2 in these cells.

3.3.3. Overexpression of RTK antagonists block the morphological but not all molecular changes induced by FGF—Lens epithelial cells transduced with RTK antagonists were treated with a differentiating dose (50ng/ml) of FGF for 5 days. Epithelial cells transduced with either the control (Ad5EGFP) or mutant Spry2 (Ad5-Y55A-Spry2) elongated with FGF stimulation over the 5 day culture period (Fig. 5B, J), compared to control cells not treated with FGF that retained their cobblestone shape (Fig. 5A,I). In Spry1- and Spry2-overexpressing explants (Fig. 5D,D′,F,F′) the majority of cells failed to elongate in response to FGF, with the exception of some cells (Fig. 5D,F, arrows). Cells transduced with Spred2 and treated with FGF (Fig. 5H) showed a similar phenotype to cells transfected with Spry family proteins, with most cells failing to elongate (Fig. 5H′). Overall, the overexpression of different RTK-antagonists was able to effectively block FGF-induced lens epithelial cell elongation and differentiation into fibers, consistent with our transfection studies. When we examined for β-crystallin labeling in the FGF-treated explants, we noticed strong reactivity in the elongated cells transduced with control (Ad5EGFP) and mutant Spry2 (Ad5-Y55A-Spry2) adenoviruses (Fig. 5K,O), as well as Ad5Spry1-, Ad5Spry2- or Ad5Spred2-expressing cells (Fig. 5L-N). Immunoblotting for levels of β-crystallin in Ad5Spry2- and Ad5-Y55A-Spry2 -expressing cells treated with FGF followed by densitometric analysis showed no significant difference in levels of β-crystallin when standardised against GAPDH levels (Fig. S3; student's *t*-test; $p>0.6$).

3.3.4. Overexpression of Spry suppressed FGF-induced expression of Prox1—We then examined the explants for Prox1 expression, a marker of lens fiber differentiation that has recently been shown to be dependent on ERK1/2-signaling (Audette et al., 2016). We compared the levels of Prox1 in both Spry2- and mutant Y55A-Spry2 -transduced epithelial explants treated with FGF over a 10 hr period. In control explants (Y55A-Spry2), Prox1 levels increased from 1 hr (Fig. 7), remaining elevated over the 10 hr culture period (Fig. 7B,C). In epithelial cells transduced with Spry2 and treated with FGF, the levels of Prox1 remained unchanged over the culture period (Fig. 7A). Quantitative analysis confirmed that Prox1 levels in Y55A-Spry2 overexpressing cells increased significantly over the culture period and this was significantly suppressed in Spry2 overexpressing cells (Fig. 7C, student's *t*-test; $p< 0.05$).

4. Discussion

FGFs have been well known to promote the morphologic and molecular changes to lens throughout embryogenesis and postnatal growth, not only establishing but maintaining its distinctive architecture and polarity throughout life (Chamberlain and McAvoy, 1989; Chamberlain and McAvoy, 1997; Lovicu and McAvoy, 2005; Zhao et al., 2015). The MAPK/ERK1/2-signaling pathway has also been shown to be important in lens development and growth (Upadhya et al., 2013), more specifically in regulating FGF-induced lens epithelial cell proliferation and fiber differentiation (Lovicu and McAvoy 2001; Le and Musil 2001; Iyengar et al., 2006, 2007; Wang et al., 2009). In turn, ERK1/2-signaling has been shown to be negatively regulated by RTK antagonists including Spry, Sef and Spred (Hacohen et al., 1998; Casci et al., 1999; Kramer et al., 1999; Chambers and Mason, 2000; Wakioka et al., 2001; Li et al., 2003; Ozaki et al., 2005), all of which are expressed in lens through eye and lens development (Boros et al., 2006; Newitt et al., 2010; Zhao et al., 2015). Given the overlapping expression of these different antagonists in lens, it was important to investigate their respective functions in regulating lens cellular processes.

Earlier studies from our laboratory using transgenic mice overexpressing murine Sef demonstrated the ability of Sef to effectively block the elongation and differentiation of lens fiber cells (Newitt et al., 2010), primarily through its reported ability to compromise FGFR-signaling (Tsang et al., 2002). Given that Sef-deficient mice did not present a lens phenotype (see Newitt et al., 2010), this led us to turn our focus on the function of other antagonists found in lens, including Spry and Spred. Spry and Spred are also recognized as negative regulators of the RTK signaling pathway; however, recent studies show that in postnatal lenses deficient for Spry1/2, there is a resultant increase in ERK1/2 phosphorylation. This led to aberrant Smad2-signaling, contributing to an epithelial to mesenchymal transition of the lens epithelium resulting in cataract (Shin et al., 2012). While this is more fitting of Spry modulating serine-threonine receptor signaling (typical of TGFβ, for example), overexpression of Spry1/2 in lens negatively regulated phosphorylation of ERK1/2 and this led to a disruption to lens cell proliferation and fiber differentiation (Shin et al., 2015), similar to that seen in lenses of mice deficient for MAPK1 (ERK2; see Upadhya et al., 2013).

Based on this, the current study primarily used an *in vitro* approach to compare the efficacy of different members of both the Spry and Spred families in regulating FGF-induced lens fiber differentiation. Here we demonstrate an inhibitory role for the different Spry and Spred members in regulating lens fiber differentiation in vitro, with overexpression of Spry or Spred family members in rat lens epithelial explants resulting in a reduced ability of cells to undergo fiber differentiation in response to FGF.

4.1. Overexpression of RTK antagonists in lens blocks FGF-induced cell elongation

To overexpress the different RTK antagonists of interest in primary lens epithelial cells, we initially used liposome-mediated transfection. Despite only a low transfection rate we could still assess individually transfected cells and readily calculate the percentage of these cells undergoing morphological changes. Following transfection, epithelial cells in the negative control groups expressing only the reporter gene underwent elongation when cultured with

FGF over 5 days, highlighting that the expression of EGFP does not impair the ability of these cells to respond to FGF. Moreover, the overexpression of a Spry2 missense-mutant (Y55A-Spry2) also did not affect the ability of cells to respond to FGF and elongate, supporting that the Spry Tyr55 residue is required for its RTK antagonist function.

When lens epithelial cells were transfected with the specific RTK antagonists, there was a significant reduction in the percentage of elongated cells following treatment with FGF; however, approximately a third of transfected cells were still able to elongate, consistent with earlier studies (see Shin et al., 2015). While we cannot rule out that the different antagonists may not be able to completely block FGF-induced cell elongation, the inability to block all cell elongation could relate to some of the cells expressing insufficient levels of the respective antagonist, something we could not control for, nor validate. Based on this we used adenoviral delivery to increase gene transfer efficiency in lens explants.

4.1.1. Overexpression of Spry inhibits FGF-induced lens fiber differentiation—

After adenoviral-mediated transduction of primary lens epithelia, control transduced (Ad5EGFP) cells readily responded to FGF stimulation and elongated, similar to epithelial cells transduced with the non-functional Spry2 (Ad5-Y55A-Spry2). In contrast, both Spry1 and Spry2 inhibited the majority of cells undergoing FGF-induced elongation and lens differentiation.

Our findings highlighting the negative regulatory ability of Spry is consistent with results in earlier studies. In PC12 pheochromocytoma cells, neurite outgrowth has been reported to be activated by via FGF/nerve growth factor (NGF)-induced Ras signaling (Hagag et al., 1986; Szeberenyi et al., 1990; Wood et al., 1992). In recent studies by Gross et al. (2001), approximately 80% of PC12 cells overexpressing Spry1 or Spry2, treated with FGF/NGF for 3 days, were blocked in their ability to differentiate, with shorter neurites and their reduced branching, compared to control PC12 cells not expressing Spry (Gross et al., 2001). When the lens epithelium from gain-of-function (GOF) transgenic mice that overexpress Spry1 or Spry2 ($Spry1^{GOF}$ or $Spry2^{GOF}$) was isolated and cultured with FGF (Shin et al., 2015), we see findings consistent with our present study where fiber cell elongation and differentiation is blocked. In these transgenic mice overexpressing Spry1 or Spry2, a ‘smaller’ lens phenotype was reported, as lens fiber cell elongation/differentiation was compromised, being shorter compared to those in WT lens (Shin et al., 2015). There was also a significant reduction in the rate of lens epithelial cells differentiating into fibers in these transgenic mice, especially in the $Spry2^{GOF}$ mice. Given there were still some relatively normal fiber cells observed in the $Spry1^{GOF}$ and $Spry2^{GOF}$ mice (Shin et al., 2015), this suggested that overexpression of Spry is not able to completely block fiber differentiation in all cases, consistent with results from the present study.

4.1.2. Spry negatively regulates FGF-induced ERK1/2 signaling and Prox1

ERK1/2: The adenoviral transfection approach labeled the majority of cells in lens epithelial explants, and allowed us to further characterize these tissues not only at the morphologic level, but for changes in the expression of different protein markers using western blotting. Spry family members have been reported to negatively regulate FGF-

induced ERK1/2 activation (Casci et al., 1999; Hacohen et al., 1998; Kramer et al., 1999). ERK1/2 phosphorylation levels in Spry2-overexpressing cells treated with FGF were consistently decreased compared to the mutant Spry2 (Y55A-Spry2) cells. This is consistent with our other *in vitro* studies where lens epithelial explants prepared from $Spry1^{GOF}$ or $Spry2^{GOF}$ mice demonstrated a marked reduction in ERK1/2 phosphorylation induced by FGF compared to WT tissue (Shin et al., 2015). Moreover, recent studies highlighted the same inhibitory effects of Spry on ERK1/2 signaling in gastric cancer cells, myeloma cells and in chronic lymphocytic leukemia (Xu et al., 2017; Tsavachidou et al., 2004; Shukla et al., 2016).

Prox1: The reduced differentiation response in Spry2-overexpressing cells cultured in FGF, was accompanied with lower Prox1 expression for up to 10 hours. Prox1 has been reported to be important for fiber elongation in mouse lens (Wigle et al., 1999; Audette et al., 2016), and it has been demonstrated to be regulated by FGF-signaling (Zhao et al., 2008;; Audette et al., 2016). In lens of mice lacking FGFR1-3, Prox1 is expressed at lower levels (Zhao et al., 2008) and shows a phenotype similar to Prox1-null mice lenses (Zhao et al., 2008; Audette et al., 2016), including impaired elongation of fiber cells leading to apoptosis (Wigle et al., 1999). This may be related to the suppressed level of pERK1/2 as seen in our explants, given that reduced levels of phosphorylated ERK1/2 was also found in Prox1-deficient lenses compared to WT lenses (Audette et al., 2016). In rat lens explants cultured with a differentiating dose of FGF, a strong label for Prox1 was associated with fiber differentiation, and this Prox1 upregulation was attenuated in the presence of either an antagonist of FGFR-signaling or an inhibitor of the ERK1/2 pathway, but not the PI3K/Akt-signaling inhibitor (Audette et al., 2016), indicating that ERK1/2-signaling is required for Prox1 expression. In the present study, in our primary cells overexpressing the mutant non-functional Spry2, Prox1 levels were shown to increase in response to FGF, and these cells elongated normally, consistent with the higher levels of phosphorylated ERK1/2. In contrast, in cells overexpressing Spry2, both phosphorylated ERK1/2 and Prox1 were reduced. While the reduced Prox1 levels observed in Spry2 overexpressing cells in our study may account for the impaired FGF-induced cell elongation, this does not necessarily account for the relatively normal accumulation of β-crystallin in these cells, of which Prox1 is thought to regulate (see Audette et al., 2016). pERK1/2-signaling has previously been shown to regulate Prox1 but not β-crystallin in lens explants (Lovicu and McAvoy, 2001; Audette et al., 2016); consistent with the current findings; however, in embryonic lenses deficient for Prox1, there is a loss of β-crystallin accompanying the block to primary fiber cell elongation (see Audette et al., 2016). This lack of concordance between our β-crystallin and Prox1 data may be attributed to putative differences between the regulatory role of Prox1-induced β-crystallin expression in primary fibers compared to secondary fiber differentiation of which we examine here. We can also not rule out that Prox1 may only be transiently repressed in our treated cells, similar to what we observed for pERK1/2-signaling (previously shown to regulate Prox1; see Audette et al., 2016). With further detailed characterization of Prox1 levels, and that of many other related regulatory genes, for example c-Maf, we may begin to better understand the molecular mechanisms regulating the processes associated with the differentiation of primary and secondary lens fiber cells.

4.1.3. Overexpression of Spred inhibits FGF-induced lens fiber differentiation—In our transfection studies, we showed for the first time that overexpression of Spred1, Spred2 or Spred3 also significantly reduced the ability of lens epithelial cells to elongate and differentiate in response to FGF stimulation; consistent with Spreds in other studies. In PC12 pheochromocytoma cells overexpressing Spred1 or Spred2 for example, cell differentiation was reduced upon treatment with NGF (Wakioka et al., 2001). Our data suggests that the three Spred family members have some differential suppression effects on FGF-induced fiber cell differentiation, with Spred2 the most potent inhibitor compared to Spred1 and Spred3. While Spred3 appeared more active than Spred1, there was no significant difference in their inhibitory ability. Although we do not show how Spred proteins mechanistically prohibit lens epithelial cell elongation into fiber cells, the primary mode of regulation is also thought to be by blocking ERK1/2 phosphorylation, like Spry. As we propose for Spry proteins, the variable suppression effects of the different Spreds in our model may simply be due to inherent properties of the cells and their differential expression of the RTK antagonists.

There is a strong inverse correlation between the levels of Spreds and Ras-MAPK activity (Wu et al., 2000; Wakioka et al., 2001). In HEK293 cells, EGF activation of Elk-1, a nuclear transcription factor targeted by MAPKs, was suppressed by overexpression of Spred1 or Spred2, in a dose-dependent manner (see Wakioka et al., 2001). C2C12 cells differentiate into promyocytes when cultured in differentiation media for up to 5 days, with MAPK activity in these cells decreasing rapidly by day 2 (Bennett and Tonks, 1997; Wu et al., 2000) to allow the formation of myotubes (Wakioka et al., 2001). During this period of ‘lower’ MAPK activity, increased levels of Spred1 are detected in the C2C12 cells; however, by day 3, when the level of MAPK activity was shown to recover and increase, Spred1 levels decreased accordingly (Wakioka et al., 2001).

4.1.4. β-crystallin expression in Spry- and Spred-overexpressing epithelial cells—Using our adenoviral transfected cells, western blotting and quantitative analysis showed that the fiber-specific β-crystallin was still expressed after treatment with FGF for 5 days in Spry2-overexpressing cells, with no significant difference when we compared cells expressing the non-functional mutant Spry2. Immunofluorescent labeling of the lens epithelial cells transfected with different adenoviruses (Ad5Spry1, Ad5-Y55A-Spry2 and Ad5Spred2) confirmed that β-crystallin was still present in all cells cultured in the presence of FGF, regardless of the fact that many failed to elongate. Previous studies by our laboratory showed that increased levels of Sef expression in the lens of transgenic mice impaired fiber cell elongation and differentiation, but did not disrupt the accumulation of β- and γ-crystallins in these cells (Newitt et al., 2010). Similarly, when rat lens epithelial explants were treated with the MEK1/ERK1/2-signaling inhibitor, UO126, FGF-induced fiber cell elongation is blocked; however, these epithelial-like cells continue to accumulate β-crystallin (see Lovicu and McAvoy, 2001). In lens explants isolated from Spry1- or Spry2-overexpressing transgenic mice treated with FGF, we see that the reduced ability of cells to elongate into fiber cells does not impair expression of β-crystallin (Shin et al., 2015). Collectively, from these studies it is evident that FGF-induced β-crystallin accumulation associated with fiber differentiation, is not dependent on ERK1/2-signaling. Our current

studies not only support this, but indicate that Spry and Spred inhibit ERK1/2-signaling responsible for the elongation of cells. Spry proteins' suppressive effects on FGF-induced ERK1/2 activation has previously been reported (Casci et al, 1999; Hacohen et al., 1998), supported by *in situ* suppression of endogenous ERK1/2 phosphorylation levels in lens cells of transgenic mice overexpressing either Spry1 or Spry2 (Shin et al., 2015). As discussed earlier, the expression of β-crystallin associated with FGF-induced differentiation of postnatal lens epithelial cells may be independent of Prox1, given that Prox1 expression is dependent on pERK1/2-signaling and not PI3K/Akt-signaling (see Audette et al., 2016), the inverse to what we see for FGF-induced β-crystallin (see Wang et al., 2009).

4.1.5. Conclusion—Using different transfection techniques we demonstrated that all the RTK antagonists examined have a potential inhibitory role in the regulation of FGF-induced fiber cell elongation associated with differentiation. This is consistent with the fact that many of these antagonists are primarily expressed in the lens epithelium and are downregulated as primary fibers mature and secondary fiber cells elongate and differentiate in situ (see Boros et al., 2006; Newitt et al., 2010; Zhao et al., 2015). While our transfection studies demonstrated that Spry1 and Spred2 were more effective in blocking FGF-induced lens epithelial cell elongation, we cannot exclude that this effectiveness may be dependent on the level of gene expression in specific cells that we could not control for.

We conclude that the RTK antagonists negatively regulate the ERK1/2 pathway, and through this may directly or indirectly interfere with other pathways such as PI3K/Akt signaling, potentially impacting on cell viability. The establishment of the adenoviral delivery system to transduce primary lens epithelial cells with high efficiency, proved advantageous in that we could better characterise cell responsiveness at the molecular level; with demonstrable reductions in ERK1/2 phosphorylation concomitant with decreased fiber-specific Prox1 labeling; however, with no noticeable reductions in β-crystallin levels. This highlighted that Spry and Spreds are primarily targeting ERK1/2 signaling-dependent cell elongation associated with lens fiber differentiation, with multiple signaling pathways required for lens fiber differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Overexpression of Sprouty or Spred members in lens epithelial cells can suppress FGF-induced fiber elongation/differentiation
- Spry1 and Spred2 are most effective at blocking FGF-induced fiber cell elongation in this in vitro system
- Spry-activity suppresses phosphorylation of ERK1/2
- Overexpression of Spry blocks ERK1/2-dependent Prox1, but not ERK1/2-indpenedent β-crystallin expression

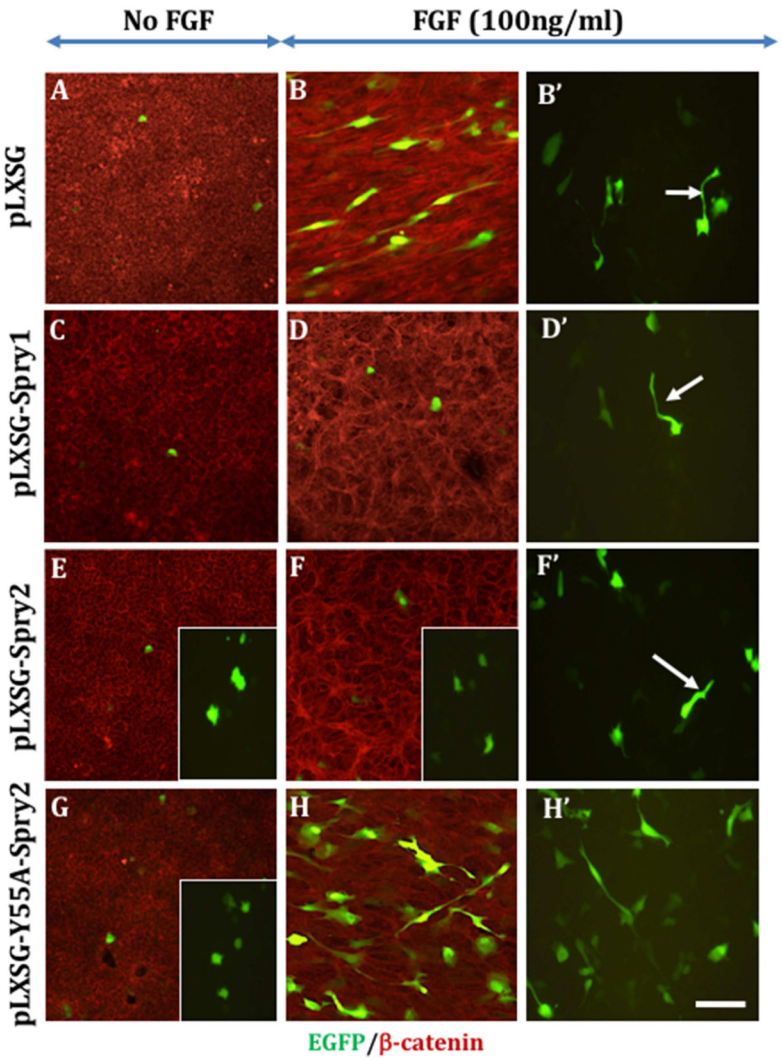

Fig. 1. Inhibition of FGF-induced fiber cell elongation by Spry

Representative micrographs of P10 rat lens epithelial explants transfected (Lipofectamine®) with pLXSG (control, A, B and B′), pLXSG-Spry1 (C, D and D′), pLXSG-Spry2 (E, F and F′) or pLXSG-Y55A-Spry2 2 (control, G, H and H′), and cultured with or without FGF (100ng/ml) for 5 days. β-catenin immunolabeling (red) outlined the cell membranes. Arrows indicate cells with a fiber cell morphology. Higher magnification insets are shown. Scale bar: 50μm, insets, 25 μm.

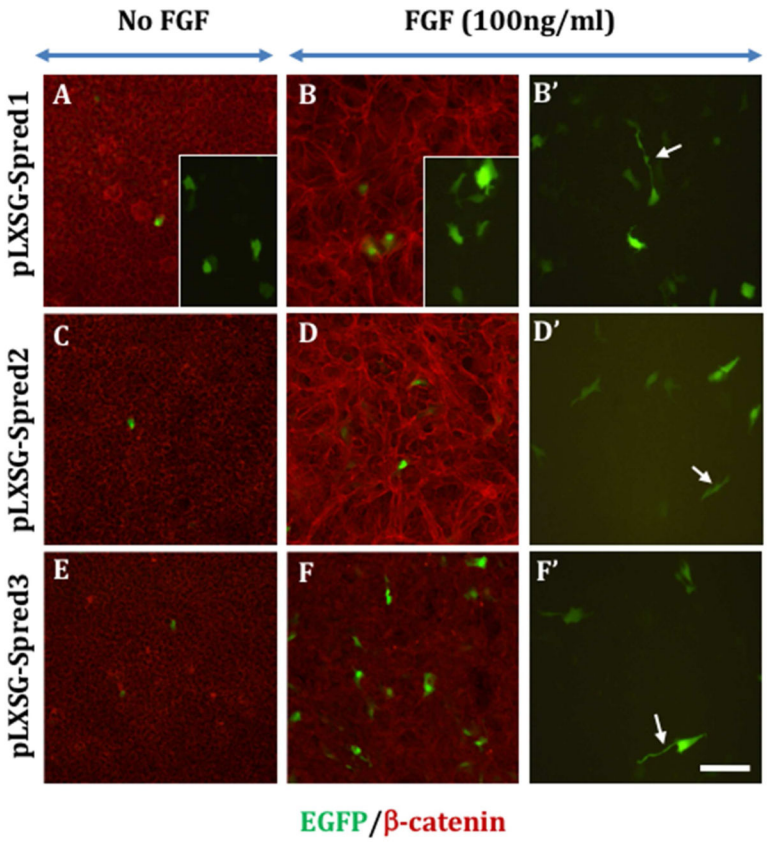

Fig. 2. Inhibition of FGF-induced fiber cell elongation by Spred

Representative micrograph of P10 rat lens epithelial explants transfected with pLXSG-Spred1 (A, B and B′), pLXSG-Spred2 (C, D and D′) or pLXSG-Spred3 (E, F and F′) using Lipofectamine®, and cultured with FGF (100ng/ml, B, B′, D, D′, F and F′) or without FGF (A, C and E) for 5 days. β-catenin immunolabeling (red) outlined the cell membranes. Arrows indicate cells with a fiber cell morphology. Higher magnification insets are shown. Scale bar: 50μm, insets, 25μm.

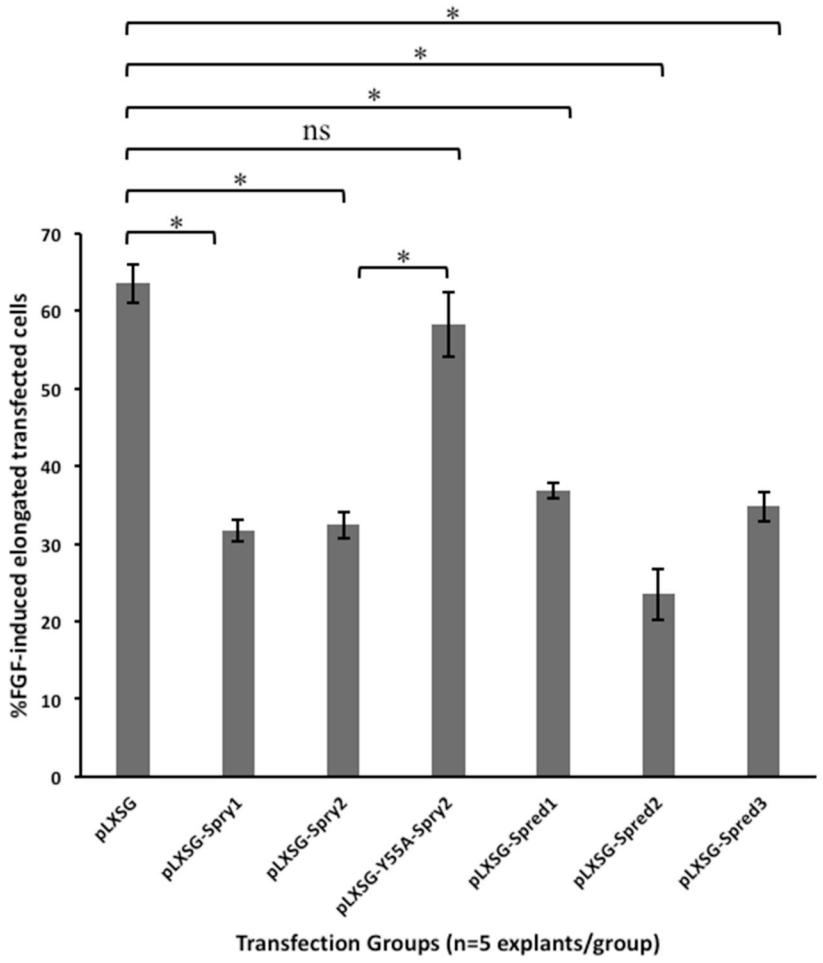

Fig. 3. Spry and Spred inhibit FGF-induced elongation of lens epithelial cells

EGFP-positive cells from the transfected lens explants (n=5 explants/group) were scored based on cell morphology. Data represents mean ± s.e.m with statistical tests performed using student's *t*-test (*=$p<0.001$; ns=not significant).

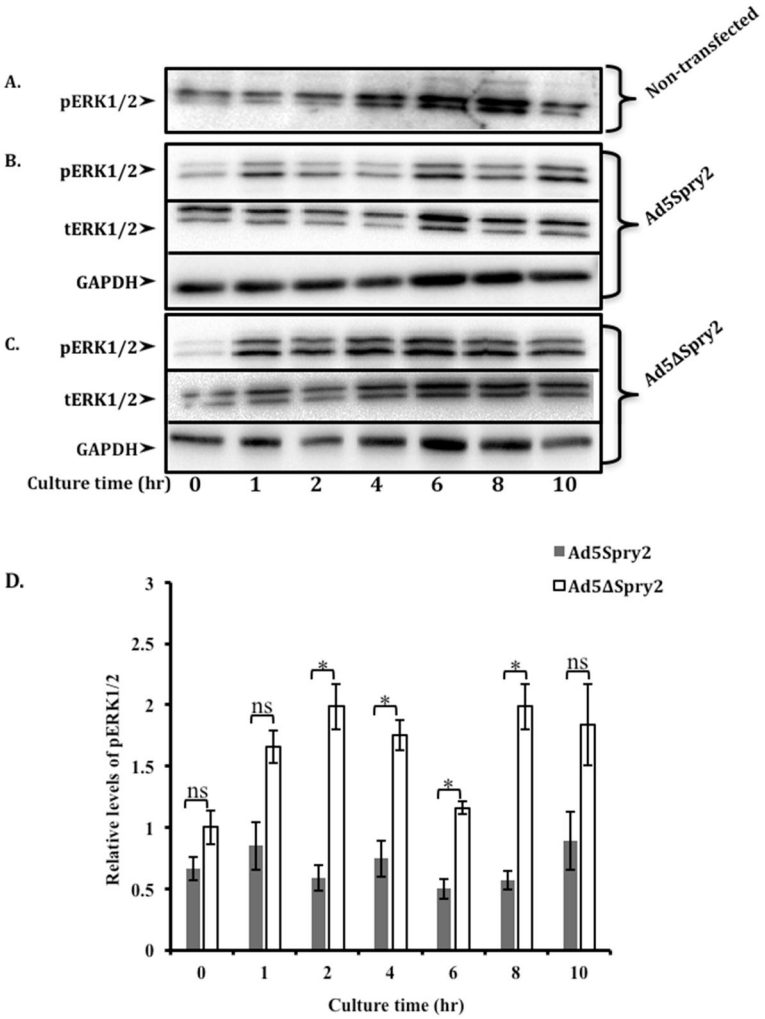

Fig. 4. Spry negatively regulates FGF-induced ERK1/2 phosphorylation

Immunoblotting of phosphorylated ERK1/2 from non-transduced FGF-treated P10 rat lens epithelial explants (A) or transduced with Ad5Spry2 (B) or Ad5-Y55A-Spry2 (C). Representative western blots of phosphorylated (p)- or total (t)-ERK1/2 with GAPDH loading control. (D) Quantitative analysis of pERK1/2 levels in Spry2- and Y55A-Spry2-overexpressing cells. Both pERK1/2 and tERK1/2 were standardised to GAPDH, with the ratio of pERK1/2 against tERK1/2 in Y55A-Spry2-overexpressing cells at 0 min set to a value of 1, against which all other ratios were determined. Data represents mean ± s.e.m with statistical tests performed using student's *t*-test (*=$p<0.001$; ns=not significant).

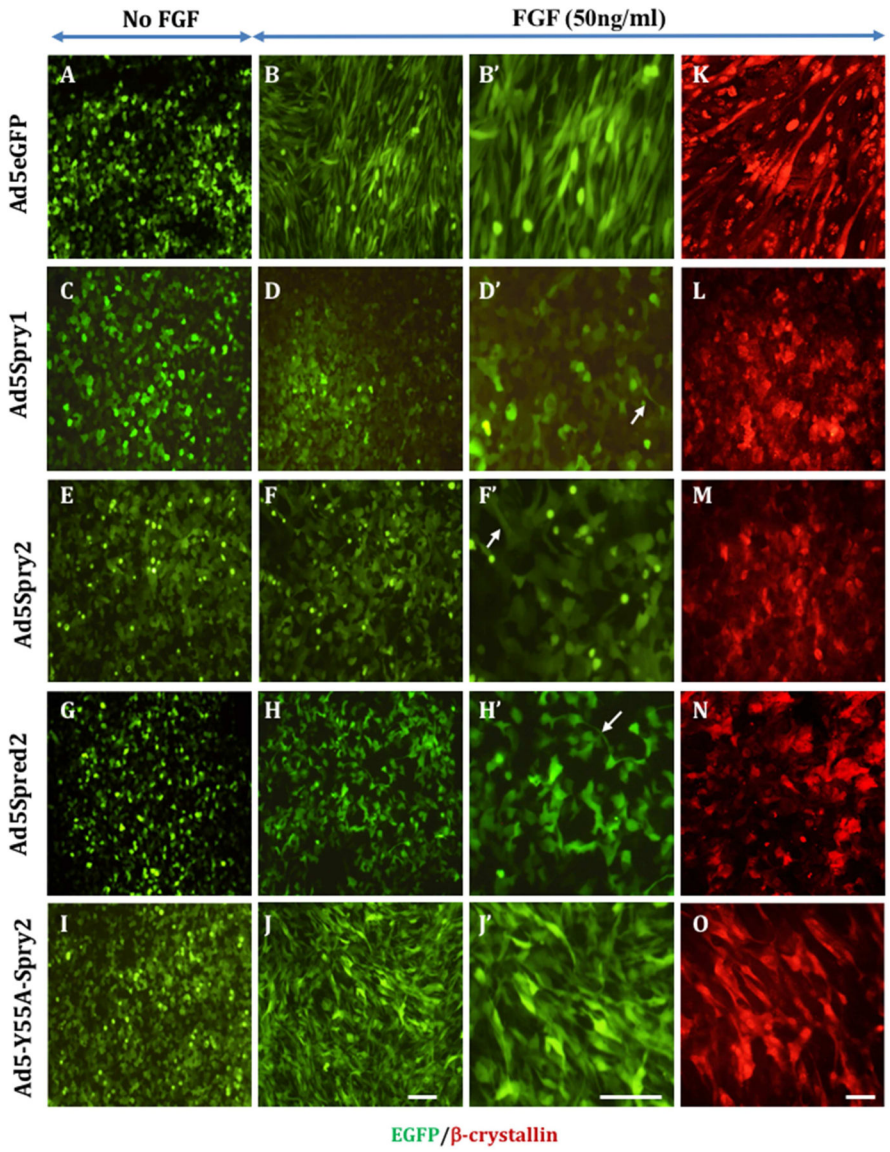

Fig. 5. Inhibition of FGF-induced fiber cell elongation by Spry or Spred

Representative micrographs of P10 rat lens epithelial explants transfected with Ad5eGFP (control; A, B, B′ and K), Ad5Spry1 (C, D, D′ and L), Ad5-Y55A-Spry2 (E, F, F′ and M), Ad5Spred2 (G, H, H′ and N) or Ad5-Y55A- Spry2 (control; I, J, J′ and O), and then treated with no FGF (A, C, E, G and I) or with 50 ng/mL FGF (B, B′, D, D′, F, F′, H, H′, J, J′, K-O) for 5 days. β-crystallin staining is shown in K-O. Arrows indicated elongate cells. Scale bar: 50 μm.

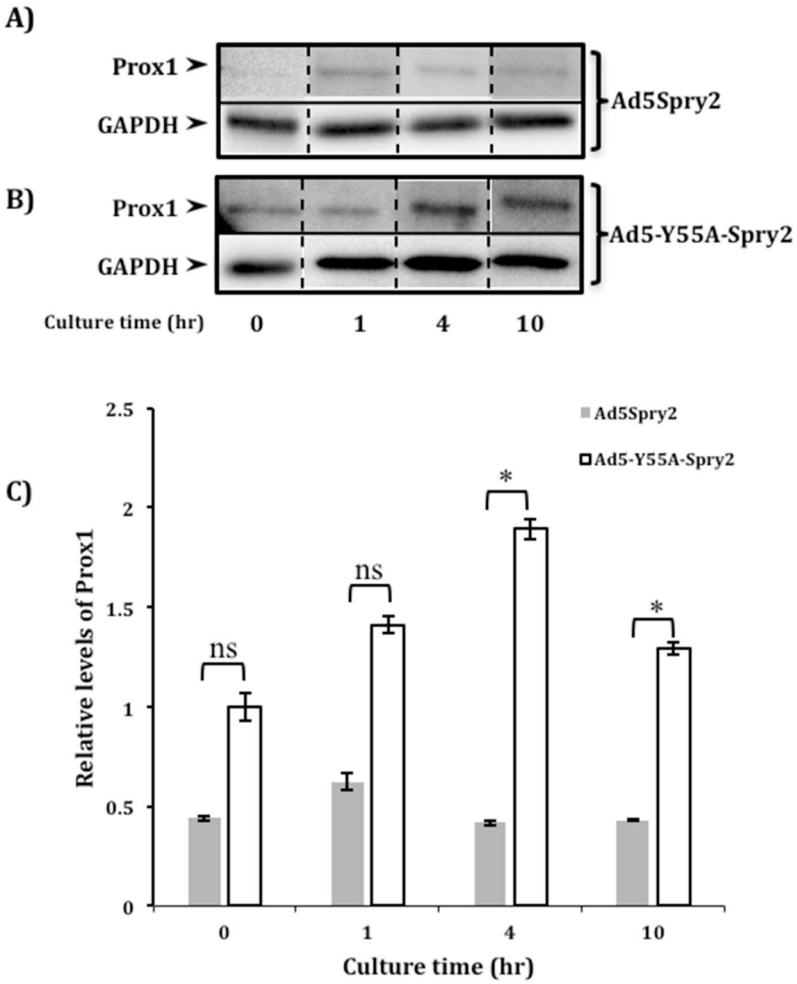

Fig. 6. Spry suppression of FGF-induced Prox1 expression

Representative western blots for Prox1 (upper panel) and GAPDH (loading control; lower panel) from lysates of P10 rat lens epithelial explants overexpressing Spry2 (A) or Y55A-Spry2 (B), and cultured with FGF for 0 to 10 hrs. (C) Quantitative analysis of western blots of Prox1 (normalised to GAPDH). Data represents mean ± s.e.m with statistical tests performed using student's *t*-test (*=p<0.05; ns=not significant).