

Sprouty Is a Negative Regulator of Transforming Growth Factor β -Induced Epithelial-to-Mesenchymal Transition and Cataract

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Fibrosis affects an extensive range of organs and is increasingly acknowledged as a major component of many chronic disorders. It is now well accepted that the elevated expression of certain inflammatory cell-derived cytokines, especially transforming growth factor β (TGF β), is involved in the epithelial-to-mesenchymal transition (EMT) leading to the pathogenesis of a diverse range of fibrotic diseases. In lens, aberrant TGF β signaling has been shown to induce EMT leading to cataract formation. Sproutys (Sprys) are negative feedback regulators of receptor tyrosine kinase (RTK)-signaling pathways in many vertebrate systems, and in this study we showed that they are important in the murine lens for promoting the lens epithelial cell phenotype. Conditional deletion of *Spry1* and *Spry2* specifically from the lens leads to an aberrant increase in RTK-mediated extracellular signal-regulated kinase 1/2 phosphorylation and, surprisingly, elevated TGF β -related signaling in lens epithelial cells, leading to an EMT and subsequent cataract formation. Conversely, increased *Spry* overexpression in lens cells can suppress not only TGF β -induced signaling, but also the accompanying EMT and cataract formation. On the basis of these findings, we propose that a better understanding of the relationship between *Spry* and TGF β signaling will not only elucidate the etiology of lens pathology, but will also lead to the development of treatments for other fibrotic-related diseases associated with TGF β -induced EMT.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2012.00111

INTRODUCTION

An epithelial-mesenchymal transition (EMT) involves multiple molecular and morphologic changes to a polarized epithelial cell that enables it to assume a mesenchymal cell phenotype (1). This specific process, which typically involves changes to how cells interact with their basement membrane, results in increased cell migratory activity as well as enhanced production, deposition and contraction of the extracellular matrix (ECM; 2–5). EMT can enable such cell transdifferentiation in normal processes beginning in early development, such as gas-

trulation and neural crest migration (6–7), as well as during organogenesis and persisting into adulthood, in which EMT not only promotes myofibroblast formation in a range of tissues, but also has been implicated in invasion and metastasis of many epithelial cancers (8–12). Under epithelial stress, such as inflammation or tissue injury, EMT is involved in wound healing, scarring and fibrogenesis (13–15), with prolonged EMT (characterized by an extensive deposition of ECM and tissue remodeling; 16–18) leading to tissue fibrosis, organ failure and death (4).

Transforming growth factor- β (TGF β) has been the most intensively studied regulator of EMTs in both normal and pathological conditions (19). Given the increasing complexity of TGF β -mediated signaling, our understanding of the required mechanisms leading to EMT is becoming more complex. The canonical TGF β -Smad signaling cascade can cross-talk with other signaling pathways, such as receptor tyrosine kinase (RTK)-mediated pathways including mitogen-activated protein kinase (MAPK) pathways, the Rho-like GTPase signaling pathway and the phosphatidylinositol-3-kinase/Akt pathway (20–21). Members of the Sprouty (*Spry*; 22), *Sef* (similar expression to *fgf*; 23) and *Spred* (*Sprouty* related EVH1 domain-containing protein; 24) families have all been identified and characterized as repressors of the RTK-mediated Ras/MAPK/extracellular signal-regulated kinase (ERK) pathway (25–28). *Spry*, for example, first identified in *Drosophila* as a negative regulator of

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Submitted March 11, 2012; Accepted for publication April 10, 2012; Epub (www.molmed.org) ahead of print April 11, 2012.

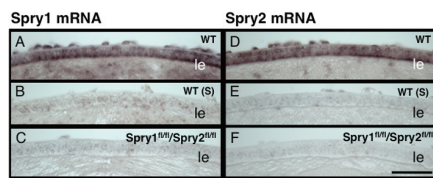


Figure 1. Expression of Spry1 and Spry2 transcripts in the neonatal mouse lens. Representative micrographs of *in situ* hybridization labeling for Spry1 mRNA (A–C) or Spry2 mRNA (D–F) using antisense (A, C, D, F) or sense (B, E) digoxigenin-labeled riboprobes, on WT lenses (A, B, D, E) or transgenic lenses deficient for Spry1 and Spry2 (Spry1^{fl/fl}/Spry2^{fl/fl}) (C, F, using MLR10-Cre). le, lens epithelium. Scale bar, 50 μ m.

fibroblast growth factor (FGF) in tracheal branching morphogenesis (22) is highly conserved, with human (*SPRY1-SPRY4*) and murine (*Spry1-Spry4*) homologues (22,29–31). In general, these molecules have been reported to negatively regulate growth factor-induced cellular proliferation, migration and differentiation (32–35). In mammals, Spry proteins inhibit RTKs, such as receptor-mediated signaling for FGF (32,36), glial cell line-derived neurotrophic factor (GDNF/RET; 37–38) and platelet-derived growth factor (32,35). In some cellular contexts, however, Spry has also been known to potentiate RTKs, such as in EGFR (endothelial growth factor receptor) signaling (39–40). Spry function is directed at various levels of the Ras/MAPK pathway, with approximately a dozen interacting partners identified, including Grb2, FRS2, Raf-1 and Shp2 (27,41).

Recent evidence has emerged implicating Spry proteins in inhibiting EMT characteristics. In lung cancer models *in vitro*, Spry4 has been shown to reverse the EMT phenotypes of tumor cells (42). Moreover, Spry downregulation may be required for the progression of TGF β -induced EMT and fibrosis, because Spry2 has been shown to be downregulated in fibrotic lung fibroblasts (43–45). Consistent with this finding, Spry seems to have an inverse relationship with TGF β ,

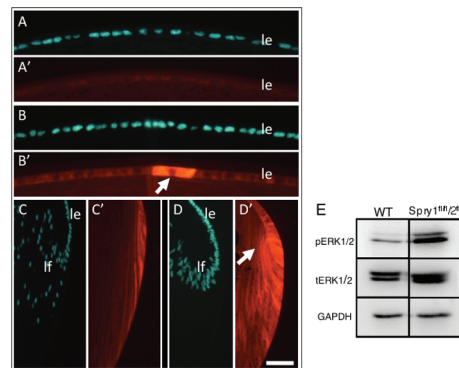


Figure 2. Spry-deficient lenses display increased immunolabeling for ERK1/2 (pERK1/2) phosphorylation in the postnatal mouse lens. Representative anterior (A, A', B, B') and equatorial (C, C', D, D') sections of postnatal-d-21 WT (A, A', C, C') and Spry-deficient (B, B', D, D') lenses using MLR10-Cre, immunolabeled for pERK1/2 (A'–D') or counterstained with Hoechst dye (A–D). Compared to the WT lens, Spry-deficient lenses displayed stronger labeling for pERK1/2 in the epithelial (le) and secondary fiber cells (lf), especially at the lens equator (D', arrow), with increased labeling also throughout the more anterior central lens epithelium, with very strong labeling in isolated cells (B', arrow). (E) Western blotting of cell lysates from postnatal-d-15 WT (left panels) or Spry-deficient (Spry1^{fl/fl}/2^{fl/fl}, right panels) lenses (n = 60), immunolabeled for either pERK1/2 (top panels), total ERK1/2 (tERK1/2, middle panels) or GAPDH (lower panels). Spry-deficient lenses demonstrated stronger labeling for pERK1/2 compared with WT lenses. Scale bar, A, A', B, B' = 25 μ m; C, C', D, D' = 65 μ m.

with Spry transcripts downregulated in response to TGF β in human lens epithelial cells (46) and in mesenchymal cells (47). Furthermore, Spry1, Spry2 and Spry4 are downregulated in a variety of cancer types, including breast, prostate, liver and lung cancers, especially in metastatic malignant stages involving EMT (42,48–50). Taken together, Spry proteins are considered to possess tumor-suppressing ability (42,51–53).

To better understand the relationship of Spry in TGF β -induced EMT, we have adopted the eye lens system. The ocular lens is an excellent model to study cell behavior because the lens is avascular, is not innervated, and has a simple, yet highly ordered, organization of two distinct cell types (54). The lens is made up of an anterior monolayer of lens epithelial cells that overlie a mass of elongate, precisely aligned fiber cells, all surrounded by a thick basement membrane, the lens capsule (55). Any disruption to this lens architecture leads to loss of lens transparency, namely cataract. Numerous studies have shown that inappropriate

TGF β signaling in the eye results in an EMT that bears a morphologic and molecular resemblance to some forms of human cataract, including anterior sub-capsular cataract (ASC) and posterior capsular opacification (PCO; 56). Reports of increased levels of TGF β in many eye diseases (57), together with other studies *in vitro* and *in vivo*, have shown that TGF β -induced EMT of lens epithelial cells results in human ASC phenotypes (57,58–64). All report the presence of elongate, spindle-shaped myofibroblast-like cells, as well as lens capsule wrinkling, apoptotic cell death and extensive accumulation of ECM, interspersed between the transdifferentiated cells. In the present study we report that conditional loss of Spry in the postnatal lens results in deregulation of TGF β signaling in the lens epithelium, resulting in an EMT and subsequent cataract. Conversely, we show that overexpression of Spry in lens cells can effectively block TGF β -mediated signaling, in turn blocking EMT and cataract formation. Although the specific signaling pathways and

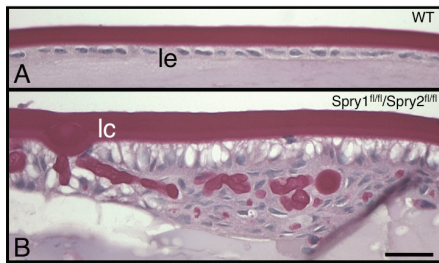


Figure 3. Spry-deficient murine lenses develop anterior subcapsular plaques. Representative histological sections of postnatal-d-80 WT mouse lenses (A) or mouse lenses deficient for all alleles of Spry1 and Spry2 (Spry^{fl/fl}/Spry2^{fl/fl}, B) using MLR10-Cre, stained with periodic acid Schiff reagent. The anterior monolayer of central lens epithelial cells (le) of WT mice (A) becomes multilayered in the absence of Spry (B), with cells closest to the restructured lens capsule (lc) appearing vacuolated. The more posterior cells acquire a spindle-shaped morphology as they deposit PAS-reactive ECM to form an anterior subcapsular plaque. Scale bar, 30 μ m.

mechanisms influenced by Spry in the lens are yet to be determined, a better understanding of these molecular interactions may serve to facilitate the development of putative therapeutic agents for the prevention of cataract and other fibrotic diseases *in situ*.

MATERIALS AND METHODS

Animals

All animal procedures conformed to the Association for Research in Vision and Ophthalmology Incorporated Resolution on the Use of Animals in Ophthalmic Research and were carried out in accordance with the Animal Care Ethics Committee at the University of Sydney (NSW, Australia).

Different inbred transgenic lines of mice were generated or obtained for this study. For our Cre-recombinase-mediated deletion of Sprouty studies, we used *Sprouty1* and *Sprouty2* floxed mouse lines (37,65–66) together with MLR10-Cre (67) or Le-Cre (68) transgenic mouse lines, to generate different allelic

combinations of *Spry1* and *Spry2* in the lens (MLR10-Cre) or multiple ocular tissues, including the lens (Le-Cre). In all cases, animals were mated so that all Cre-positive progeny inherited only one copy of the Cre-recombinase transgene. Polymerase chain reaction (PCR) primers used to ascertain for the expression of Cre, the recombination and excision of loxP flanked sequences in Sprouty 1 (*Spry1*) and Sprouty 2 (*Spry2*) genes, have been described previously (37,65,67–68).

Murine Spry1 was overexpressed in transgenic mice, specifically in the lens, by use of a modified α A-crystallin promoter, fused to a chick δ 1-crystallin enhancer element, a rabbit β -globin intron and a human growth hormone polyA signal (69). Mice transgenic for Spry1, specifically in the lens, were mated to our transgenic TGF β lines (64,70), to overexpress Spry1 in this specific background. As previously reported, for genotyping, PCR primers specific to the δ 1-crystallin enhancer element (69) or the SV40 polyadenylation region (64) were used to genotype our Spry1- or TGF β -transgenic lines, respectively. All transgenic animals characterized (that is, Cre, Spry1, TGF β) were hemizygous for their respective transgene.

Histology and Immunolabeling

Postnatal murine tissues at different ages (ranging from neonatal to postnatal d 80) were fixed in 10% neutral buffered formalin for histological processing and subsequent periodic acid Schiff (PAS) staining and immunolabeling. Six-micron-thick sections of paraffin wax-embedded tissues were used for all analyses. For immunofluorescent labeling of α -smooth muscle actin (α -sma), phosphorylated Smad2 (pSmad2), Snai1 and Snai2 tissue sections were hydrated to phosphate-buffered saline (PBS) supplemented with bovine serum albumin (BSA), and incubated with 3% (volume/volume) normal goat serum before application of primary antibody. Sections were treated overnight at 4°C with antibodies for α -sma (diluted 1:100; Sigma-

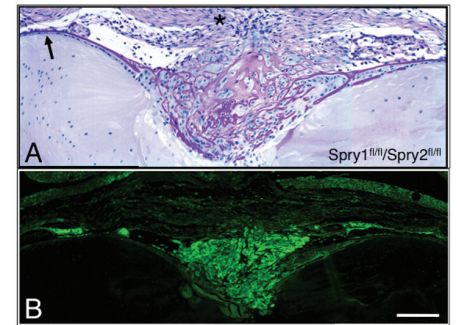


Figure 4. Spry-deficient murine lenses develop anterior subcapsular plaques with evidence of an EMT. Representative histological sections of postnatal-d-26 mouse lenses deficient for all alleles of Spry1 and Spry2 (Spry^{fl/fl}/Spry2^{fl/fl}) using Le-Cre, stained with PAS reagent (A) or immunolabeled for α -sma (B). Lenses deficient for Spry develop a distinct anterior subcapsular plaque (A) with evidence of an EMT as numerous α -sma-labeled myofibroblastic cells (B) lay down, and are embedded within, a meshwork of extracellular matrix extending from the lens capsule. The lens capsule appears relatively normal on either side of the centrally located fibrotic plaque (A, arrowhead). Note that the lens tissue is attached anteriorly to the overlying Spry-deficient corneal tissue (A, asterisk), an association persisting from embryogenesis. Scale bar, 100 μ m.

Aldrich, Castle Hill, NSW, Australia), pSmad2 (diluted 1:50; Calbiochem, San Diego, CA, USA), Snai1 (diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or Snai2 (diluted 1:50; Santa Cruz Biotechnology). Following application of secondary antibodies; anti-mouse Alexa-fluor 488 (Invitrogen/Molecular Probes, Eugene, Oregon, USA; diluted 1:1000 for α -sma) or anti-rabbit Cy-3 antibody (Sigma; diluted 1:200 for pSmad2, Snai1, Snai2) sections were counterstained with bisbenzimidazole (Hoechst dye; Sigma 33342) at 2 μ g/mL and mounted with 10% PBS/glycerol.

For immunofluorescent labeling of phosphorylated forms of ERK1/2 (pERK) or E-cadherin, hydrated tissue sections were first subjected to heat-induced (105°C) antigen retrieval in

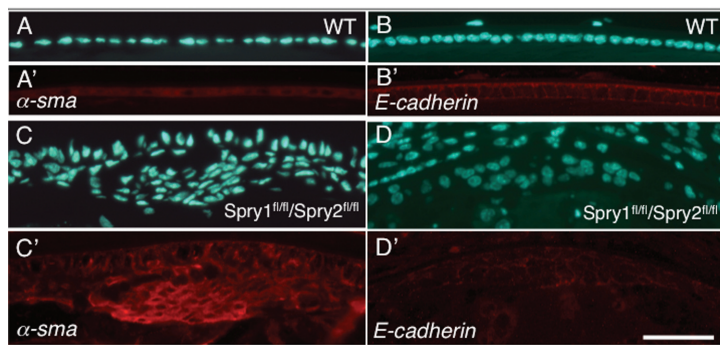


Figure 5. Epithelial cells from Spry-deficient murine lenses undergo an EMT, resulting in anterior subcapsular fibrotic plaques. Representative sections of postnatal-d-80 (A, A', C, C') or postnatal-d-10 (B, B', D, D') WT mouse lenses (A, A', B, B') or lenses deficient for all alleles of Spry1 and Spry2 (Spry1^{fl/fl}/Spry2^{fl/fl}) by using either MLR10-Cre (C, C') or Le-Cre (D, D'), immunolabelled for either α -sma (A', C') or E-cadherin (B', D') with cell nuclei counterstained with Hoechst dye (A–D). Compared to WT lens epithelia that are nonreactive for α -sma (A'), lens cells deficient for Spry express α -sma, undergoing an EMT to form anterior subcapsular plaques (C'). Consistent with this finding, the normal membrane distribution of E-cadherin in lens epithelial cells of WT mice (B') is reduced and lost in Spry-deficient cells that multilayer as they undergo an EMT (D'). Scale bar, 50 μ m.

10 mmol/L sodium citrate (pH 6) for 10 min, before cooling for 20 min at room temperature and application of 3% normal goat serum (71). The anti-phospho-ERK1/2 (1:100; Cell Signaling, Danvers, MA, USA) and E-cadherin (1:200; Invitrogen, Carlsbad, CA, USA) antibodies were subsequently applied overnight at 4°C and their binding was detected with a goat anti-rabbit Cy3-conjugated secondary antibody (diluted 1:200; Sigma). Sections were counterstained with Hoechst dye as described above.

Immunoblotting for pERK1/2 was based on methods previously described (72). Postnatal lenses were homogenized with a micropestle in 1.5-mL tubes containing 2.5 mmol/L EDTA (ethylenediaminetetraacetic acid), 25 mmol/L Tris-HCL (pH 7.5), 0.375 mol/L NaCl, 1% IGEPAL (octylphenoxypolyethoxyethanol), 1.5 mmol/L sodium orthovanadate and a protease inhibitor cocktail (Roche, Basel, Switzerland). Following protein estimation, 20 μ g of each protein sample was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred onto a polyvinylidene difluoride membrane and immunolabeled for either phosphorylated ERK1/2 (anti-

rabbit, 1:2000; Cell Signaling), total ERK1/2 (anti-rabbit, 1:2000, Cell Signaling) or glyceraldehyde 3-phosphate dehydrogenase (anti-mouse, 1:20000; HyTest, Turku, Finland) by use of enhanced-chemiluminescence substrate (Millipore, Billerica, MA, USA).

In situ hybridization of Spry1 and Spry2 mRNA transcripts was carried out as previously described (73), with sense and antisense riboprobes labeled with digoxigenin. Immunolabeling with an anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche) was used to detect the distribution of the hybridized riboprobes.

Preparation of Lens Epithelial Explants

Eyes were collected from 15-d-old mice, in medium 199 with Earle's salts (M199) supplemented with 0.1mg/mL L-glutamine, 50 IU/mL penicillin, 50 mg/mL streptomycin, 2.5 mg/mL Amphostat B (all from Trace Scientific, Victoria, Australia) and 0.1% BSA (Sigma). Lens epithelial explants were prepared as described previously (74).

To induce EMT, lens explants were exposed to varying concentrations of human recombinant TGF β -2 (25 pg/mL

to 200 pg/mL; R&D Systems, Minneapolis, MN, USA). All treated explants were cultured for up to 5 d at 37°C in 5% CO₂. At the end of the culture period, explants were fixed in 10% neutral buffered formalin for 20 min and stored in 70% ethanol before immunolabeling for α -sma (75). To better delineate cells in our explants, lens epithelial explants were labeled for 10 min for lectin binding with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated Triticum vulgare lectin (30 μ g/mL; Sigma) to stain cell membranes or with 67 ng/mL of propidium iodide (Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature to label cell nuclei (76).

Imaging

Whole lenses were photographed by using phase-contrast microscopy, histological sections were visualized under bright field illumination, and immunofluorescent-labeled sections or explants were visualized with epifluorescence (Leica DMLB, Wetzlar, Germany) or confocal microscopy (Zeiss LSM5; Carl Zeiss, North Ryde, NSW, Australia), respectively.

All supplementary materials are available online at www.molmed.org.

RESULTS

The primary aim of this study was to conditionally delete *Spry1* and *Spry2* in the ocular lens of transgenic mice, to assess their role in lens development and growth. Using a Cre-*loxP* approach, we showed by *in situ* hybridization that both *Spry1* and *Spry2* were effectively deleted from all neonatal mouse lens cells (Figure 1; also see Supplementary Figure S1). For this experiment, transgenic mice carrying floxed alleles of *Spry1* and *Spry2* (37,77) were mated to mice of two different transgenic lines expressing Cre-recombinase in the developing lens. One transgenic line expressed Cre-recombinase specifically to the lens under the control of a modified α crystallin promoter (MLR10-Cre; 67). The other line, adopting a modified Pax6 promoter (Le-Cre; 68),

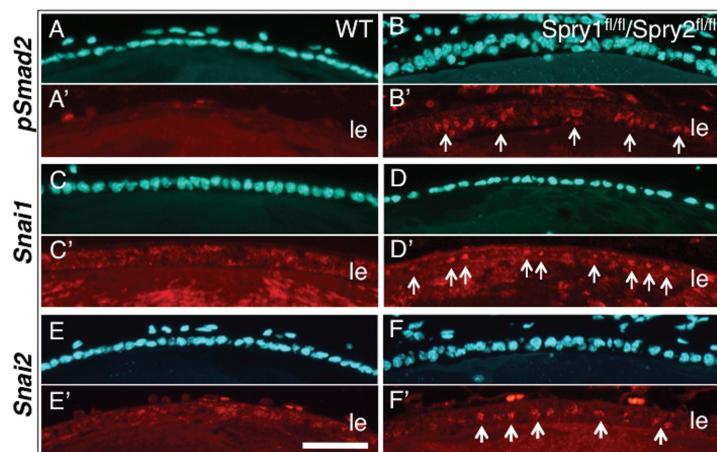


Figure 6. Epithelial cells from *Spry*-deficient murine lenses display TGF β -like signaling. Representative sections of neonatal WT mouse lenses (A, A', C, C', E, E') or lenses deficient for all alleles of *Spry1* and *Spry2* (*Spry*^{fl/fl}/*Spry2*^{fl/fl}) by using Le-Cre (B, B', D, D', F, F'), immunolabelled for either pSmad2 (A', B'), Snai1 (C', D') or Snai2 (E', F'), with cell nuclei counterstained with Hoechst dye (A–F). Compared to WT lens epithelia (le) that do not display nuclear reactivity for pSmad2 (A'), Snai1 (C') or Snai2 (E') lens cells deficient for *Spry* demonstrate a clear nuclear label for all of these downstream TGF β -mediated signaling molecules (B', D', F'; arrows), prior to cell multilayering to form anterior subcapsular plaques. Scale bar, 50 μ m.

strongly expressed Cre-recombinase in ocular cells of the anterior segment, including lens, cornea, conjunctiva and eyelid precursors. In the first part of this study we characterized and reported on lines of mice deficient for *Spry1* and *Spry2* in the lens using both of these systems. Given that all the findings on the lens were similar when we used either of these Cre-lines to knock out *Spry* in this tissue, representative results from each line are presented throughout.

Because *Spry1* and *Spry2* are reported to negatively regulate the Ras-ERK1/2-MAPK–signaling pathway, we used immunofluorescent labeling and Western blotting to search for changes in the levels of phosphorylated ERK1/2 (pERK1/2) in postnatal-d-2 *Spry*-deficient lenses. Compared to wild-type (WT) mouse lenses (Figures 2A, A', C, C'), pERK1/2 was markedly elevated in epithelial cells and secondary fiber cells of lenses of *Spry*-deficient mouse lenses (Figures 2B, B', D, D'). This was supported by using Western blotting, comparing pERK1/2 levels in WT lenses with lenses deficient for *Spry1* and *Spry2* (Figure 2E). Quantifi-

cation of pERK1/2 levels by using densitometry of these Western blot bands displayed an approximate 3-fold increase in pERK1/2 in *Spry*-deficient lenses compared with WT mouse lenses (data not shown).

Spry-Deficient Lenses Display EMT Leading to Anterior Subcapsular Plaques

By postnatal d 80, *Spry*-deficient lenses developed cataracts, primarily due to a prominent anterior subcapsular fibrotic plaque derived from the lens epithelial cells (Figure 3). Compared to the WT lens (Figure 3A), the epithelial cells of lenses deficient for all four alleles of the *Spry1* and *Spry2* (using MLR10-Cre) multilayer acquired a spindle-shaped morphology (Figure 3B). The cells closest to the lens capsule were vacuolated, and aberrant deposition of PAS-stained ECM was deposited throughout the plaque. This accumulation of ECM around these myofibroblast-like cells, together with the restructuring of the adjacent lens capsule, is consistent with the lens epithelial cells undergoing an EMT. A similar

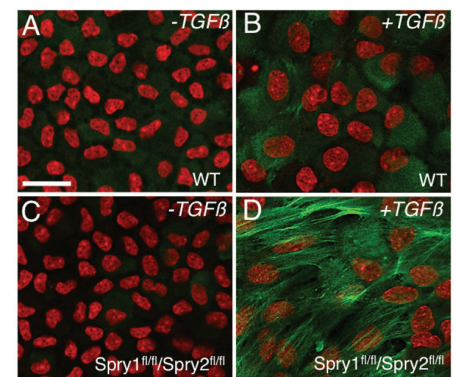


Figure 7. *Spry*-deficient lens epithelial cells are more responsive to TGF β -induced EMT. Epithelial explants prepared from postnatal-d-15 WT murine lenses (A, B) or lenses deficient for all alleles of *Spry1* and *Spry2* (*Spry*^{fl/fl}/*Spry2*^{fl/fl}) by using MLR10-Cre (C, D) were exposed to either no TGF β (A, C) or 200 pg/mL TGF β (B, D) for 24 h before immunolabelling for α -sma (green) and counterstaining cell nuclei with propidium iodide (red) (A–D). *Spry*-deficient lens epithelial cells were more sensitive to the effects of TGF β , with greater immunolabeling for α -sma (D) compared with WT lens cells (B) that express lower endogenous *Spry* levels. Scale bar, 20 μ m.

EMT-like phenotype was also evident in the lens when we used the other Cre-recombinase–expressing line (Le-Cre) to delete all alleles of *Spry1* and *Spry2* (Figure 4). In these lines of mice, the anterior subcapsular plaques are well established by postnatal d 26, with the central lens epithelial cells also becoming spindle shaped and depositing aberrant ECM (Figure 4A). These embedded cells are strongly reactive for α -sma (Figure 4B), a marker for myofibroblasts and EMT. It should be noted that a deficiency of *Spry* in both the lens and corneal epithelial precursors using Le-Cre resulted in the attachment of the cornea to the lens via a persistent lens stalk. Once established during embryogenesis, this close association remains throughout postnatal eye development and growth. This ocular phenotype has been reported and characterized in more detail elsewhere (78). We cannot exclude the fact that this retention

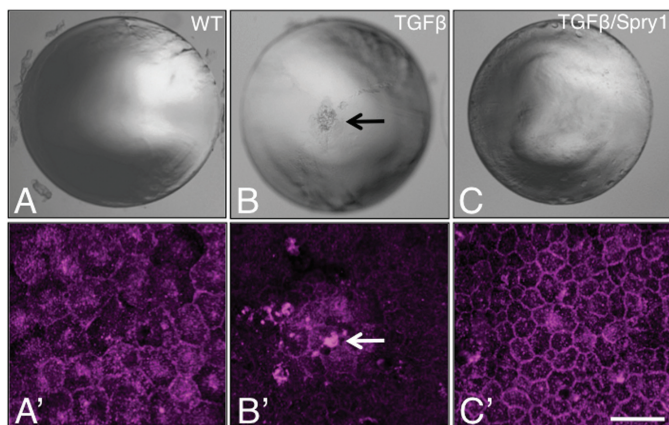


Figure 8. Spry can prevent TGF β -induced ASC formation. Representative phase-contrast images of whole lenses (A–C) or epifluorescent images of lens epithelial whole mounts, with cell membranes stained with TRITC-conjugated lectin (A'–C'), from postnatal-d-15 lenses from either (A, A') WT mice, (B, B') transgenic mice overexpressing the mature form of human TGF β 1 specifically to the lens (TGF β) or (C, C') transgenic mice overexpressing TGF β 1 as well as Spry1 specifically to the lens (TGF β /Spry1). In contrast to the transparent lens (A) and uniform monolayer of lens epithelia (A') from WT mice, lenses from transgenic mice overexpressing TGF β 1 develop anterior subcapsular cataracts (B, arrowhead), reflected by the disruption to the ordered lens epithelium (B', arrow). Increasing the levels of Spry1 specifically in lenses of transgenic mice overexpressing TGF β 1 prevents the formation of anterior subcapsular cataracts (C), with the retention of a monolayer of ordered lens epithelial cells (C'). Scale bar, A'–C' = 20 μ m.

of a lens stalk contributes to or exacerbates the lens phenotype leading to cataract formation in these Spry1^{fl/fl}/Spry2^{fl/fl}-Le-Cre lines of mice.

Lens-specific deletion of all Spry alleles using either Le-Cre or MLR10-Cre led to anterior subcapsular cataract formation. The major difference between the two was that cataracts were observed much earlier in the Spry1^{fl/fl}/Spry2^{fl/fl}-Le-Cre lines of mice compared with the Spry1^{fl/fl}/Spry2^{fl/fl}-MLR10-Cre lines of mice. It should be also noted that when fewer alleles of Sprouty are deleted, for example all alleles of *Spry2* (Spry1^{+/+}/Spry2^{fl/fl}-MLR10-Cre lines), a similar, albeit less severe, cataractous phenotype results (see Supplementary Figure S2B). In the case of only *Spry1* deletion (Spry1^{fl/fl}/Spry2^{+/+}-MLR10-Cre lines), there was no evidence of anterior subcapsular plaques (see Supplementary Figure S2A). When both *Spry1* and *Spry2* were deleted, regardless of the Cre-line used, the lens cells demonstrated similar features at both the morphologic and

molecular level. For example, compared with WT lens epithelial cells that do not normally express α -sma (Figures 5A, A'), the myofibroblastic cells of the Spry-deficient lenses using MLR10-Cre also accumulated α -sma (Figures 5C, C'). Changes to other markers involved in EMT progression, such as the dispersion and loss of E-cadherin, were also evident in postnatal-d-80 Spry-deficient lenses (Figures 5D, D') when compared with the normal membrane-associated labeling of E-cadherin in the intact epithelium of WT lenses (Figures 5B, B').

Spry-Deficient Lenses Promote TGF β Signaling

Given that elevated TGF β activity in the eye has been shown to induce a similar EMT in the lens, leading to anterior subcapsular cataract formation (56) such as that seen in Spry-deficient lenses, we assayed for markers of TGF β signaling (nuclear localization of pSmad2, Snai1 and Snai2) in neonatal Spry-deficient lenses, prior to disruption of the lens

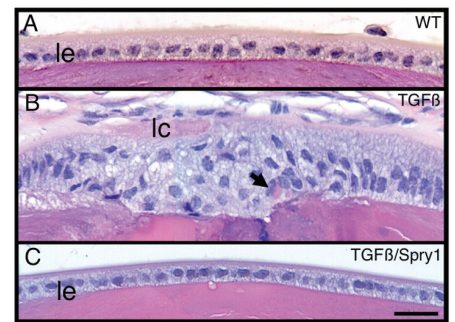


Figure 9. Spry can prevent TGF β -induced anterior subcapsular plaque formation. Representative histological sections of postnatal-d-10 (A) WT mouse lenses and transgenic mouse lenses (B) overexpressing TGF β 1 (TGF β) or (C) both TGF β 1 and Spry1 (TGF β /Spry1), stained with PAS reagent. The anterior monolayer of central lens epithelial cells (le) of WT mice (A) becomes multilayered with elevated levels of TGF β 1 (B), with elongating spindle-shaped cells reorganizing the overlying lens capsule (lc), as well as depositing PAS-reactive ECM (B, arrowhead) in the developing anterior subcapsular plaque. Increasing the levels of Spry1 specifically in lenses of transgenic mice overexpressing TGF β 1 prevents the formation of this anterior subcapsular plaque formation (C), with the lens epithelial cells remaining as a monolayer (C). Scale bar, 25 μ m.

epithelium leading to cataract formation. In neonatal WT mice we did not detect any nuclear localization of pSmad (Figures 6A, A'), Snai1 (Figures 6C, C') or Snai2 (Figures 6E, E'). In contrast, in Spry-deficient lenses, with the use of either Le-Cre or MLR10-Cre prior to lens epithelial cell multilayering there was distinctive nuclear labeling for pSmad2 (Figures 6B, B') as well as Snai1 (Figures 6D, D') and Snai2 (Figures 6F, F'), indicative of TGF β signaling.

To determine whether epithelial cells from these Spry-deficient lenses were more sensitive to the effects of TGF β , we prepared epithelial explants from lenses of 15-d-old WT and Spry-deficient (MLR10-Cre) mouse lines and cultured them in the presence of TGF β . After a 24-h culture period, TGF β -treated lens epithelial cells from WT mice underwent

an EMT and were immunoreactive for α -sma (Figure 7B), compared with cells not treated with TGF β (Figure 7A). Under the same conditions, lens epithelial cells in explants deficient for Spry demonstrated a more pronounced TGF β -induced response, with greater labeling for α -sma, associated with distinctive stress fibers (Figure 7D), indicative of a more advanced EMT response. In the absence of TGF β , cultured Spry-deficient lens epithelial cells appeared similar to cells from WT mice (Figure 7C).

Spry Can Block TGF β -Induced ASC Formation

Because a deficiency in Spry in lens cells makes them more susceptible to the effects of TGF β *in vitro* and leads to aberrant TGF β signaling and EMT, resulting in ASC *in vivo*, we tested whether overexpression of Spry specifically in the lens was sufficient to block anterior subcapsular cataract formation *in situ*. By use of an established model for ASC formation, transgenic lines overexpressing TGF β -1 specifically in the lens (OVE918; see 64) were crossed to newly established transgenic mouse lines overexpressing Spry1 specifically in the lens (LOV28). Compared to lenses from WT mice that remained transparent (Figure 8A) owing to their normal monolayer of lens epithelial cells (Figure 8A'), lenses from transgenic mice overexpressing TGF β developed a distinct anterior central opacity by postnatal d 15 (Figure 8B, arrow) relating to the disruption of the epithelium with the formation of an anterior subcapsular plaque (Figure 8B', arrow). Although increased levels of Spry1 in the lens of hemizygous LOV28 lines had little impact on normal lens morphology (data not shown), when Spry1 levels were increased in transgenic mice overexpressing TGF β , the lenses remained transparent (Figure 8C) with no indication of anterior subcapsular plaque formation, with little to no disruption to the lens epithelium (Figure 8C'). With this cross breeding, there was 100% penetrance, with no loss of transparency in lenses of any mice overexpressing Spry1, irrespec-

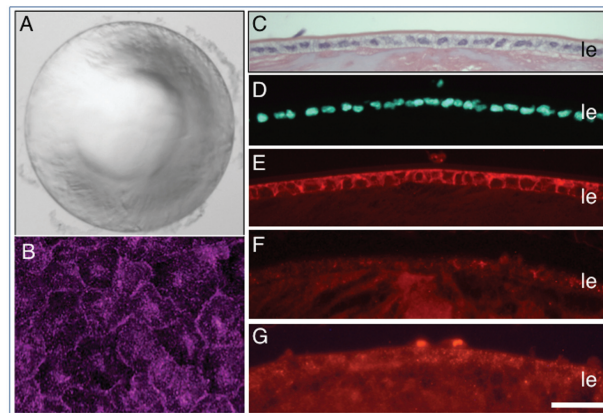


Figure 10. Representative images from postnatal-d-15 (A, B) or neonatal (C–G) Spry1-overexpressing transgenic mice depicting phase-contrast microscopy of a whole transparent lens (A), epifluorescence of an intact lens epithelial wholemount stained with TRITC-conjugated lectin (B), PAS-staining (C) or Hoechst labeling (D) of the anterior lens epithelial monolayer, strongly reactive for E-cadherin (E), but not reactive for pSmad2 (F) or Snai1 (G). le, lens epithelium. Scale bar, B = 20 μ m; C–G = 35 μ m.

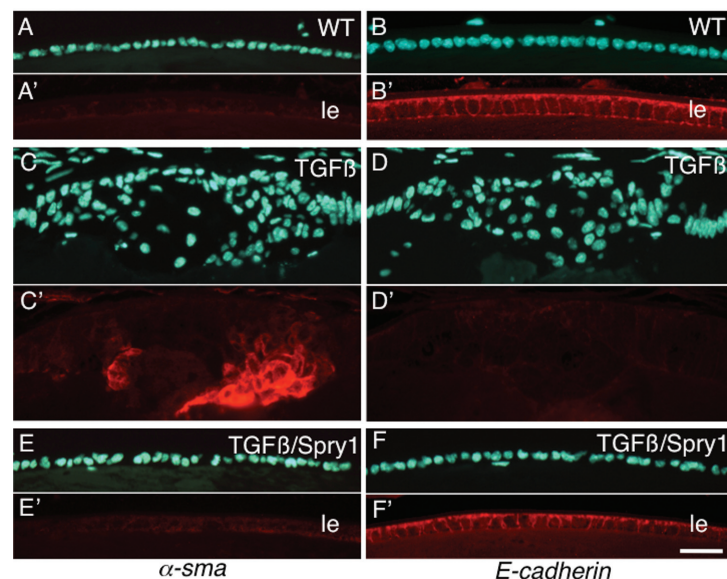


Figure 11. Spry can prevent TGF β -induced EMT leading to anterior subcapsular cataract formation. Representative sections of postnatal-d-10 WT mouse lenses (A, A', B, B') or transgenic mouse lenses overexpressing TGF β 1 (C, C', D, D'; TGF β) or both TGF β 1 and Spry1 (E, E', F, F'; TGF β /Spry1), immunolabelled for either α -sma (A', C', E') or E-cadherin (B', D', F'), with cell nuclei counterstained with Hoechst dye (A–F). Compared to WT lens epithelia (le) that are nonreactive for α -sma (A'), lens cells in transgenic mice stimulated by elevated levels of TGF β express α -sma (C') as they undergo an EMT to form anterior subcapsular plaques (C). Consistent with this observation, the normal membrane distribution of E-cadherin in lens epithelial cells of WT mice (B') is reduced and lost in transgenic mice overexpressing TGF β (D') as the cells multilayer and undergo an EMT (D). In lens epithelial cells (le) of transgenic mice overexpressing TGF β and Spry, there is no immunolabeling for α -sma (E') in this monolayer (E, F) and immunolabeling for E-cadherin (F') is retained in these cells, similar to that seen in the epithelia of WT mice. Scale bar, 20 μ m.

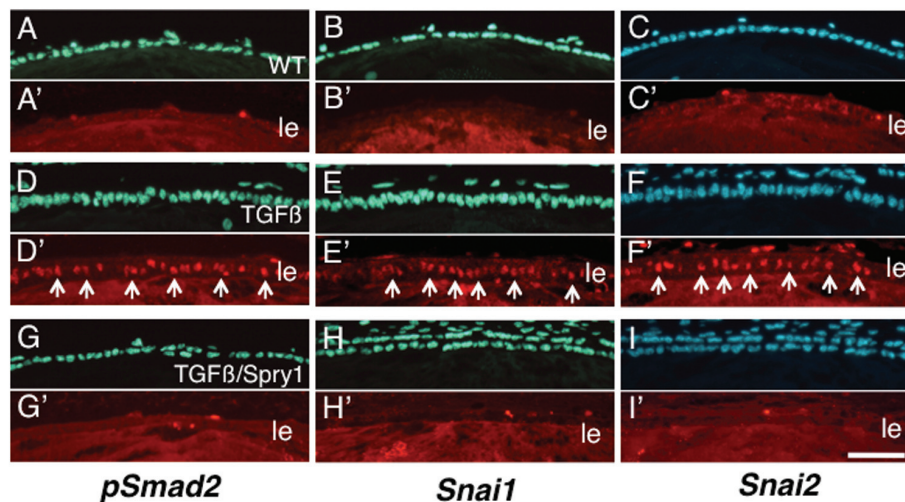


Figure 12. Spry can block TGF β -mediated signaling in lens cells. Representative sections of neonatal WT mouse lenses (A, A', B, B', C, C'), or transgenic mouse lenses overexpressing TGF β 1 (D, D', E, E', F, F'; TGF β) or both TGF β 1 and Spry1 (G, G', H, H', I, I'; TGF β /Spry1), immunolabelled for either pSmad2 (A', D', G'), Snai1 (B', E', H') or Snai2 (C', F', I'), with cell nuclei counterstained with Hoechst dye (A–I). Compared to WT lens epithelia (le) that do not display nuclear reactivity for pSmad2 (A'), Snai1 (B') or Snai2 (C'), lens cells in transgenic mice stimulated by elevated levels of TGF β demonstrate a clear nuclear label for all three of these TGF β -mediated signaling molecules (D', E', F'; arrows). In lens epithelial cells (le) of transgenic mice overexpressing TGF β and Spry, there is no nuclear labeling for pSmad2 (G'), Snai1 (H') or Snai2 (I'), similar to that seen in WT mice. Scale bar, 20 μ m.

tive of TGF β overexpression. In mice up to 21 d old, cataracts were evident only in lenses overexpressing TGF β (OVE918; n = 90) and were not present in any lenses from WT mice (n = 102), nor in mice with lenses overexpressing Spry1 (LOV28; n = 62) or coexpressing Spry1 and TGF β (n = 116). These findings were further substantiated with histological analysis of lenses from 10- and 21-d-old postnatal transgenic mice. Compared to the monolayer of lens epithelia in WT mice (Figure 9A) with overexpression of TGF β there was evident multilayering of lens epithelial cells, accompanied by aberrant PAS-stained ECM and lens capsule remodeling (Figure 9B), all hallmarks of TGF β -induced EMT and ASC progression. In contrast, lenses overexpressing Spry1, in the presence of TGF β , retained a normal monolayer of lens epithelium as well as an intact lens capsule (Figure 9C). Note that transgenic mice hemizygous for Spry1 do not develop lens opacities, consistent with their main-

tenance of the monolayer of lens epithelia (Figures 10A–C).

Spry Can Block TGF β -Induced EMT

Given that the morphological changes accompanying TGF β -induced ASC formation were blocked in the presence of Spry1, we examined whether the molecular markers associated with TGF β -induced EMT, namely the appearance of α -sma and the loss of E-cadherin, could also be blocked by Spry1 *in situ*. As demonstrated earlier, in WT mice lens epithelial cells do not express α -sma (Figures 11A, A'), and E-cadherin is evenly expressed and distributed along the epithelial cell membranes (Figures 11B, B') in these mice. With elevated levels of TGF β in lenses of transgenic mice, we see a prominent induction of EMT within 10 d of postnatal growth, because lens epithelial-derived cells multilayer to form an anterior subcapsular plaque and accumulate α -sma (Figures 11C, C'). Accompanying this process

is the progressive loss of E-cadherin in these same cells (Figures 11D, D'). When Spry1 is overexpressed, lens cells under the influence of TGF β fail to multilayer and accumulate α -sma, remaining as a monolayer (Figures 11E, E'). Consistent with this finding, the intact monolayer of lens epithelial cells is retained, as are the normal levels and distribution of E-cadherin (Figures 11F, F'). The lens epithelium of transgenic mice hemizygous for Spry1 displays normal expression of E-cadherin (see Figures 10D–E).

Spry Can Block TGF β -Signaling in the Lens

We have shown Spry to block EMT and subsequent ASC formation when highly expressed in transgenic mice overexpressing TGF β specifically in the lens. To determine whether this block to EMT and ASC formation is associated with a block to TGF β signaling, we again assayed for markers of TGF β signaling (nuclear localization of pSmad2, Snai1 and Snai2) in neonatal lenses exposed to TGF β , with or without overexpression of Spry1. As reported earlier, lenses from WT mice do not present any nuclear labeling for pSmad (Figures 12A, A'), Snai1 (Figures 12B, B') or Snai2 (Figures 12C, C'). In neonatal transgenic mice overexpressing TGF β prior to EMT and ASC formation, we see prominent labeling for pSmad (Figures 12D, D'), Snai1 (Figures 12E, E') and Snai2 (Figures 12F, F') in the nuclei of anterior lens epithelial cells. When Spry1 is overexpressed in the presence of TGF β , consistent with a block to TGF β -induced EMT (Figure 11) and ASC formation (Figures 8 and 9), there is also a block to TGF β signaling with no nuclear labeling for pSmad (Figures 12G, G'), Snai1 (Figures 12H, H') or Snai2 (Figures 12I, I') in the lens epithelium, similar to WT lenses (Figures 12A, A'–C, C'). In transgenic mice overexpressing Spry1, there is no labeling for pSmads or Snai1 (see Figures 10F, G)

Spry Can Directly Block TGF β -Induced EMT in Lens Epithelial Cells

Our *in vivo* studies have shown Spry expression results in reduced TGF β sig-

naling, leading to a block in EMT and ASC in the lens of our transgenic mice. To determine whether this antagonistic activity of Spry overexpression was directly associated with reduced sensitivity of cells to TGF β , and not through some other indirect pathway *in situ*, we compared the effects of increasing concentrations of TGF β on epithelial explants prepared from lenses from postnatal-d-15 WT mice and lenses from transgenic mice overexpressing Spry1. Note that lenses overexpressing Spry1 had up to three times the normal levels of endogenous Spry1 in the lens (data not shown). After 5 d in culture we noted that lens epithelial cells from WT mice underwent an EMT (reactive for α -sma) with as little as 25 pg/mL of TGF β 2 (Figure 13B) when compared with untreated cells (Figure 13A). With higher concentrations of TGF β 2, from 50 pg/mL and up to 200 pg/mL, this EMT became more pronounced with increasing immunoreactivity for α -sma (Figures 13C, D). When we repeated this TGF β regimen on lens epithelial explants prepared from transgenic lines overexpressing Spry1, we saw no evidence of EMT with 25 pg/mL (Figure 13F) or even 50 pg/mL (Figure 13G) of TGF β , similar to lens cells not treated with TGF β (Figure 13E). However, application of markedly higher doses of TGF β (up to 200 pg/mL) on the Spry-overexpressing lens cells was sufficient to overcome the antagonistic effects of Spry on TGF β activity, because cells underwent an EMT, with strong reactivity for α -sma evident (Figure 13H).

DISCUSSION

The distinctive architecture of the ocular lens, which determines its function, is established and maintained by defined cellular processes that are tightly regulated by the RTK-mediated signaling pathways, such as the Ras-MAPK/ERK1/2 pathway (74). These signaling pathways are in turn regulated by a multitude of intracellular antagonists including members of the Sprouty family (36,79). Given the importance of ERK1/2 signaling in mediating lens cell behavior

(80), together with the fact that Sprouty genes display very distinctive spatial expression in the lens (73), we set out to determine whether Sprouty genes are required for normal lens development and growth. Using a Cre-lox approach to conditionally delete *Spry1* and *Spry2* from the lens, we demonstrate that these genes are important for maintenance of the postnatal lens epithelium by reducing the susceptibility of these cells to ocular factors such as TGF β , which are known to contribute to lens pathology leading to cataract formation.

To effectively delete Spry from the lens we adopted two different independent lines of mice in which Cre-recombinase is driven to the lens. Using the Le-Cre lines we saw during embryogenesis a phenotype in lenses of mice deficient for *Spry1* and *Spry2*. Cre-recombinase in these lines of mice is expressed in different ocular tissues including the lens and presumptive corneal epithelium, result-

ing in the persistence of a lens stalk in the absence of Spry in both of these tissues during embryogenesis. This adhesion between the developing cornea and lens, in the absence of Spry, has been recently characterized in detail in another study (78) and will not be further addressed here. We noted, however, that postnatally lenses from these same mice developed ASC. To determine whether this lens phenotype is directly associated with the loss of Spry in the lens, and not a secondary effect of the corneal adhesion, we used a different Cre-line (MLR10-Cre) that drives Cre-recombinase specifically to the lens. We showed that this line of Spry-deficient lenses undergo similar changes to those seen in the Le-Cre line, including loss of transparency and formation of ASC; the only difference being that the pathological phenotype takes longer to materialize in the MLR10-Cre Spry-deficient lenses. This may be attributed to the persistence

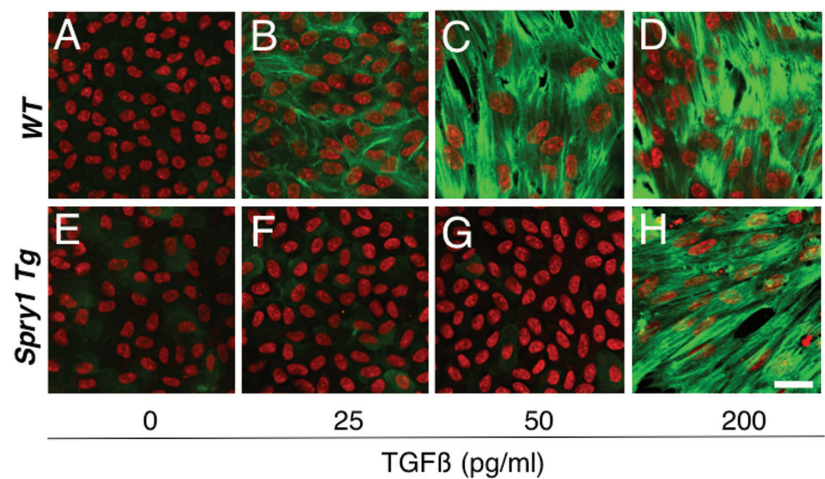


Figure 13. Spry specifically blocks TGF β -activity in lens epithelial explants. Epithelial explants prepared from postnatal-d-15 WT murine lenses (A–D) or transgenic mouse lenses overexpressing Spry1 (E–H) were exposed to no TGF β (A, E), 25 pg/mL TGF β 2 (B, F), 50 pg/mL TGF β 2 (C, G) or 200 pg/mL TGF β 2 (D, H) for up to 5 d before immunolabelling for α -sma (green), and counterstaining cell nuclei with propidium iodide (red). Epithelial cells in lens explants prepared from WT mice underwent an EMT (based on α -sma reactivity) with exposure to 25 pg/mL of TGF β (B), and the degree of this EMT was progressively more pronounced with increasing concentration of TGF β (C, D). In contrast, lens epithelial cells overexpressing Spry1 were more resistant to the effects of TGF β , with little to no response with either 25 pg/mL (F) or 50 pg/mL (G) TGF β . At much higher concentrations of TGF β (200 pg/mL), lens cells overexpressing Spry1 underwent an EMT with strong reactivity for α -sma (H), comparable to that seen in WT lens cells (D). Scale bar, 20 μ m.

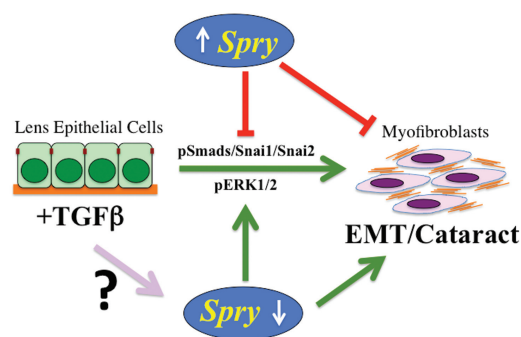


Figure 14. Influence and interaction of TGF β signaling and Spry on the epithelial phenotype, leading to the progression or prevention of an EMT in the lens.

of a lens stalk early in the development of the Spry1^{fl/fl}/Spry2^{fl/fl}-Le-Cre lines. ASC is characterized by a prominent anterior subcapsular fibrotic plaque derived from the lens epithelial cells through the process of EMT (81–82). ASC has been normally associated with conditions involving ocular inflammation or injury, such as in atopic dermatitis, uveitis and ocular surgery (83–86), and numerous studies have implicated TGF β in its pathogenesis (56,87). Similar to what we report for this study, others have shown TGF β -induced subcapsular plaques in the lens to be primarily comprised of spindle-shaped contractile myofibroblastic cells, reactive for α -sma and embedded in excessive accumulations of ECM (57,64,70,87–90). This TGF β -induced phenotype, which we now report for the first time to be present in lenses deficient for Spry, are all hallmarks of EMT. On the basis of these findings we propose that Spry has the ability to negatively regulate EMT in the lens, possibly by blocking the effects of TGF β on lens epithelia cells.

Earlier studies in other systems have implicated Spry in negatively regulating EMT. Spry levels are generally reduced in an environment involving EMT (43,47). Consistent with this observation, in nontransformed lung epithelial cell lines, knockdown of Spry4 has been shown to decrease expression of epithelial markers, with an increase in expression of mesenchymal markers (42). This finding is complemented by experiments

with overexpressed Spry4 in lung cancer cell lines. Spry4 inhibited growth of these tumor cells, as well as reversed their EMT phenotypes, with decreased cell motility and reduced vimentin expression (marker for mesenchymal state), and also promoted epithelial cell differentiation, evident by increased expression of E-cadherin (42). In the present study, we observed a similar series of events in lens cells expressing elevated levels of Spry1. Spry1 overexpression in lens cells prevented TGF β -mediated EMT that would normally result in fibrotic plaque formation and ASC. The resultant lenses were transparent and maintained an intact lens epithelium (normal cell polarity and E-cadherin levels) with no evidence of mesenchymal markers such as α -sma expression. This result was supported by *in vitro* data showing that isolated lens epithelial cells overexpressing Spry1 from transgenic mice were exposed to increasing doses of TGF β . Compared to WT lens epithelia, epithelial cells expressing higher levels of Spry1 were more resilient to the effects of TGF β , neither undergoing an EMT nor accumulating α -sma. This was not the case with higher levels of TGF β administration, because the protective effects of Spry1 could be overcome, indicating that Spry activity and effectiveness are dose dependent.

Although our *in vitro* explant studies support a role for Spry in directly blocking TGF β activity, we cannot rule out the possibility that Spry may also be acting

on other growth factor signaling pathways in the intact lens, such as FGF-mediated signaling, as has been reported in other systems (32,36). Given that FGF is known to promote ERK1/2 signaling in the lens (74) and has also been shown to exacerbate TGF β -induced cataracts *in vitro* (90), one could speculate that negative regulation of FGF signaling by Spry in lens cells may suppress anterior subcapsular cataract formation. However, given the essential role of FGF in lens development (71), the observation that our hemizygous transgenic mice overexpressing Spry1 in the lens do not present a phenotype is inconsistent with such an interpretation, although this finding may again be attributed to an insufficient dosage of Spry1.

Whether Spry's ability to prevent EMT is due directly to its ability to regulate TGF β is not yet clear, but we have demonstrated that lens epithelial cells deficient for Spry are more sensitive to TGF β compared with WT lens epithelium. This result is supported by the fact that downstream TGF β -mediated signaling molecules such as Smad2, Snai1 and Snai2 are all phosphorylated and translocated to epithelial cell nuclei in lenses deficient for Spry, similar to those seen in lenses stimulated by TGF β in our transgenic mice. If we overexpress Spry in the lenses of these TGF β -overexpressing mice, as above, we no longer see this activation and nuclear translocation of Smad2, Snai1 and Snai2; indicative of Spry influencing TGF β -mediated Smad signaling in lens epithelial cells. Sprys have not typically been reported to act on the Smad-signaling pathway, but are primarily known for their ability to block signaling pathways mediated by RTKs, including the Ras-ERK1/2 MAPK pathway. This characteristic is consistent with our Spry-deficient lines that have elevated levels of phosphorylated ERK1/2 in the lens. Although Smad-dependent signaling has not yet been shown to be directly influenced by Spry, TGF β has been reported to signal via Smad-independent pathways, such as RhoA, PI3-K/Akt and the MAPKs, including

ERK1/2, JNK (c-Jun NH₂-terminal kinase) and p38 (92–93), some of which are targets for Spry. The Ras/ERK1/2 MAP kinase cascade has been shown to be transiently activated in response to TGFβ (94–95). For example, in kidney mesangial cells, the induction of type I and IV collagen by TGFβ is dependent on ERK1/2 but not p38 MAP kinase; whereas in dermal fibroblasts this requires p38 and not ERK1/2 (96–98). These differential responses not only implicate p38 and ERK1/2 signaling cascades as profibrotic, but also highlight the complexity of interplay between the MAPK and Smad signaling pathways. There is further evidence that TGFβ/Smad signaling is tightly controlled by Ras/ERK1/2 MAPK signaling (99–100), facilitated by the fact that Smads contain ERK1/2 phosphorylation sites (99,101). In some cases these pathways cooperate, with TGFβ/Smad-induced Snai1 activity in tumor cells reported to be dependent on Ras signaling (102). These types of crosstalk are dependent on cell type, as in other systems the Ras/ERK1/2 MAPK cascade has been reported to suppress Smad-dependent responses (103). Taken together with recent studies that revealed that TGF-β1 induced EMT (in human lung cancer cells) is dependent on PI3K/Akt and MAPK/ERK1/2 signaling pathways (21), we propose that elevated ERK1/2 levels in lenses deficient for Spry may contribute to TGFβ-mediated signaling leading to EMT and subsequent cataract. This proposal is supported by the fact that ERK1/2 is activated by TGFβ in culture models of EMT and cataract (104), and inhibitors of the ERK1/2 signaling cascade can block the effects of TGFβ in different cell types (105), including the lens (106).

CONCLUSION

In summary, TGFβ-induced pSmad (and ERK1/2) signaling leads to lens epithelial cells undergoing an EMT, progressing to ASC formation. In a similar fashion, in the present study we also show that the conditional deletion of Spry specifically from the lens leads to

an aberrant increase in pERK1/2, together with elevated TGFβ-related signaling in lens epithelial cells, also leading to an EMT and subsequent ASC formation. Conversely, increased Spry expression in lens cells has the ability to suppress not only TGFβ-induced signaling, but also the accompanying EMT and ASC formation (Figure 14). We provide evidence that Spry's ability to block TGFβ is dose dependent and it remains to be determined whether TGFβ-induced EMT and cataract in the intact lens is dependent on the downregulation of Spry. A better understanding of the relationship between Spry and TGFβ signaling will not only contribute toward elucidating the etiology of lens pathology leading to cataracts such as ASC and PCO but will lead to better understanding and potentially treatment of a myriad of other fibrotic-related diseases associated with TGFβ-induced EMT (2).

ACKNOWLEDGMENTS

We would like to acknowledge G Martin (Department of Anatomy, University of California, San Francisco, USA) for providing the Sprouty flox mice, as well as the technical assistance of J Boros. The work was funded by the NIH (R01 EY03177), USA, the National Health & Medical Research Council (NHMRC), and the Sydney Foundation for Medical Research, Australia.

DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Kalluri R, Neilson EG. (2003) Epithelial-mesenchymal transition and its implications for fibrosis. *J. Clin. Invest.* 112:1776–84.
2. Leask A, Abraham DJ. (2004) TGF-β signaling and the fibrotic response. *FASEB J.* 18:816–27.
3. Wynn TA. (2007) Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J. Clin. Invest.* 117:524–29.
4. Wynn TA. (2008) Cellular and molecular mechanisms of fibrosis. *J. Pathol.* 214:199–210.
5. Kalluri R, Weinberg RA. (2009) The basics of

- epithelial-mesenchymal transition. *J. Clin. Invest.* 119:1420–28.
6. Duband JL, Monier F, Delannet M, Newgreen D. (1995) Epithelium-mesenchyme transition during neural crest development. *Acta Anat.* 154:63–78.
7. Nakaya Y, Sheng G. (2008) Epithelial to mesenchymal transition during gastrulation: an embryological view. *Devel. Growth Diff.* 50:755–66.
8. Hotz, B, et al. (2007) Epithelial to mesenchymal transition: expression of the regulators Snail, Slug, and Twist in pancreatic cancer. *Clin. Cancer Res.* 13:4769–76.
9. Larue L, Bellacosa A. (2005) Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. *Oncogene.* 24:7443–54.
10. Potenta S, Zeisberg E, Kalluri R. (2008) The role of endothelial-to-mesenchymal transition in cancer progression. *Br. J. Cancer.* 99:1375–79.
11. Vincent-Salomon A, Thiery JP. (2003) Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. *Breast Cancer Res.* 5:101–6.
12. Xu J, et al. (2006) Prostate cancer metastasis: role of the host microenvironment in promoting epithelial to mesenchymal transition and increased bone and adrenal gland metastasis. *Prostate.* 66:1664–73.
13. Desmouliere A. (1995) Factors influencing myofibroblast differentiation during wound healing and fibrosis. *Cell Biol. Int.* 19:471–76.
14. Iwano M, et al. (2002) Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J. Clin. Invest.* 110:341–50.
15. Lopez-Novoa JM, Nieto MA. (2009) Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. *EMBO Mol. Med.* 1:303–14.
16. Kim KK, et al. (2006) Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc. Natl. Acad. Sci. U. S. A.* 103:13180–5.
17. Zeisberg M, Kalluri R. (2004) The role of epithelial-to-mesenchymal transition in renal fibrosis. *J. Mol. Med.* 82:175–81.
18. Zeisberg M, et al. (2007) Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J. Biol. Chem.* 282:23337–47.
19. Zavadil J, Bottlinger EP. (2005) TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene.* 24:5764–74.
20. Zhang YE. (2009) Non-Smad pathways in TGF-beta signaling. *Cell Res.* 19:128–39.
21. Chen XF, et al. (2011) Transforming growth factor-β1 induces epithelial-to-mesenchymal transition in human lung cancer cells via PI3K/Akt and MEK/Erk1/2 signaling pathways. *Mol. Biol. Rep.* 39:3549–56.
22. Hacoen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. (1998) Sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell.* 92:253–63.

23. Tsang M, Friesel R, Kudoh T, Dawid IB. (2002) Identification of Sef, a novel modulator of FGF signaling. *Nat. Cell Biol.* 4:165–9.
24. Wakioka T, et al. (2001) Spred is a Sprouty-related suppressor of Ras signaling. *Nature.* 412:647–51.
25. Bundschu, K, Walter U, Schuh K. (2007) Getting a first clue about SPRED functions. *Bioessays.* 29:897–907.
26. Cabrita MA, Christofori G, Cabrita MA, Christofori G. (2008) Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis.* 11:53–62.
27. Mason JM, Morrison DJ, Albert Basson M, Licht JD. (2006) Sprouty proteins: multifaceted negative-feedback regulators of receptor tyrosine kinase signaling. *Trends Cell Biol.* 16:45–54.
28. Ron D, Fuchs Y, Chorev DS. (2008) Know thy Sef: a novel class of feedback antagonists of receptor tyrosine kinase signaling. *Int. J. Biochem. Cell Biol.* 40:2040–52.
29. de Maximy AA, Nakatake Y, Moncada S, Itoh N, Thierry JP, Bellusci S. (1999) Cloning and expression pattern of a mouse homologue of drosophila sprouty in the mouse embryo. *Mech. Dev.* 81:213–16.
30. Leeksa OC, et al. (2002) Human sprouty 4, a new ras antagonist on 5q31, interacts with the dual specificity kinase TESK1. *Eur. J. Biochem.* 269:2546–56.
31. Tefft JD, et al. (1999) Conserved function of mSpry-2, a murine homolog of Drosophila sprouty, which negatively modulates respiratory organogenesis. *Curr. Biol.* 9:219–22.
32. Gross I, Bassit B, Benezra M, Licht JD. (2001) Mammalian sprouty proteins inhibit cell growth and differentiation by preventing ras activation. *J. Biol. Chem.* 276:46460–8.
33. Lee CC, et al. (2004) Overexpression of sprouty 2 inhibits HGF/SF-mediated cell growth, invasion, migration, and cytokinesis. *Oncogene.* 23:5193–202.
34. Reich A, Sapir A, Shilo B. (1999) Sprouty is a general inhibitor of receptor tyrosine kinase signaling. *Development.* 126:4139–47.
35. Yigzaw Y, Cartin L, Pierre S, Scholich K, Patel TB. (2001) The C Terminus of sprouty is important for modulation of cellular migration and proliferation. *J. Biol. Chem.* 276:22742–7.
36. Hanafusa H, Torii S, Yasanuga T, Matsumoto K, Nishida E. (2002) Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signaling pathway. *Nat. Cell Biol.* 4:850–8.
37. Basson MA, et al. (2005) Sprouty1 is a critical regulator of GDNF/RET-mediated kidney induction. *Dev. Cell.* 8:229–39.
38. Basson MA, et al. (2006) Branching morphogenesis of the ureteric epithelium during kidney development is coordinated by the opposing functions of GDNF and Sprouty1. *Dev. Biol.* 299:466–77.
39. Egan JE, Hall AB, Yatsula BA, Bar-Sagi D. (2002) The bimodal regulation of epidermal growth factor signaling by human Sprouty proteins. *Proc. Natl. Acad. Sci. U. S. A.* 99:6041–6.
40. Wong ES, et al. (2002) Sprouty2 attenuates epidermal growth factor receptor ubiquitylation and endocytosis, and consequently enhances Ras/ERK signalling. *EMBO J.* 21:4796–808.
41. Kim HJ, Bar-Sagi D, Kim HJ, Bar-Sagi D. (2004) Modulation of signalling by Sprouty: a developing story. *Nat. Rev. Mol. Cell. Biol.* 5:441–50.
42. Tennis MA, et al. (2010) Sprouty-4 inhibits transformed cell growth, migration and invasion, and epithelial-mesenchymal transition, and is regulated by Wnt7A through PPAR γ in non-small cell lung cancer. *Mol. Cancer Res.* 8:833–43.
43. Renzoni EA, et al. (2004) Gene expression profiling reveals novel TGFbeta targets in adult lung fibroblasts. *Respir. Res.* 5:24.
44. Broekelmann TJ, Limper AH, Colby TV, McDonald JA. (1991) Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc. Natl. Acad. Sci. U. S. A.* 88:6642–6.
45. Sime PJ, Xing Z, Graham FL, Csaky KG, Gaudie J. (1997) Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J. Clin. Invest.* 100:768–76.
46. Dawes LJ, Elliott RM, Reddan JR, Wormstone YM, Wormstone IM. (2007) Oligonucleotide microarray analysis of human lens epithelial cells: TGFbeta regulated gene expression. *Mol. Vis.* 13:1181–97.
47. Ding W, et al. (2007) Sprouty2 downregulation plays a pivotal role in mediating crosstalk between TGF-beta1 signaling and EGF as well as FGF receptor tyrosine kinase-ERK pathways in mesenchymal cells. *J. Cell. Physiol.* 212:796–806.
48. Fong CW, et al. (2006) Sprouty 2, an inhibitor of mitogen-activated protein kinase signaling, is down-regulated in hepatocellular carcinoma. *Cancer Res.* 66:2048–58.
49. Kwabi-Addo B, et al. (2004) The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. *Cancer Res.* 64:4728–35.
50. McKie AB, et al. (2005) Epigenetic inactivation of the human sprouty2 (hSPRY2) homologue in prostate cancer. *Oncogene.* 24:2166–74.
51. Lo TL, Yusoff P, Fong CW. (2004) The ras/mitogen-activated protein kinase pathway inhibitor and likely tumor suppressor proteins, sprouty 1 and sprouty 2 are deregulated in breast cancer. *Cancer Res.* 64:6127–36.
52. Lee SA, et al. (2008) Integration of genomic analysis and in vivo transfection to identify sprouty 2 as a candidate tumor suppressor in liver cancer. *Hepatology.* 47:1200–10.
53. Shaw AT, et al. (2007) Sprouty-2 regulates oncogenic K-ras in lung development and tumorigenesis. *Genes. Dev.* 21:694–707; erratum in 2008;22:277.
54. Lovicu FJ, McAvoy JW. (2005) Growth factor regulation of lens development. *Dev. Biol.* 280:1–14.
55. McAvoy JW, Chamberlain CG, de Longh RU, Hales AM, Lovicu FJ. (1999) Lens development. *Eye.* 13:425–47.
56. Lovicu FJ, et al. (2002) TGFbeta induces morphological and molecular changes similar to human anterior subcapsular cataract. *Br. J. Ophthalmol.* 86:220–6.
57. Connor TB Jr, et al. (1989) Correlation of fibrosis and transforming growth factor-beta type 2 levels in the eye. *J. Clin. Invest.* 83:1661–6.
58. Hales AM, Chamberlain CG, Dreher B, McAvoy JW. (1999) Intravitreal injection of TGFbeta induces cataract in rats. *Invest. Ophthalmol. Vis. Sci.* 40:3231–6.
59. Hales AM, Chamberlain CG, McAvoy JW. (1995) Cataract induction in lenses cultured with transforming growth factor- beta. *Invest. Ophthalmol. Vis. Sci.* 36:1709–13.
60. Hales AM, Schulz MW, Chamberlain CG, McAvoy JW. (1994) TGF-beta 1 induces lens cells to accumulate alpha-smooth muscle actin, a marker for subcapsular cataracts. *Curr. Eye Res.* 13:885–90.
61. Liu J, Hales AM, Chamberlain CG, McAvoy JW. (1994) Induction of cataract-like changes in rat lens epithelial explants by transforming growth factor beta. *Invest. Ophthalmol. Vis. Sci.* 35:388–401.
62. Robertson JV, et al. (2007) Adenoviral gene transfer of bioactive TGFbeta1 to the rodent eye as a novel model for anterior subcapsular cataract. *Mol. Vis.* 13:457–69.
63. Saika S, et al. (2004). Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. *Am. J. Pathol.* 164:651–63.
64. Srinivasan Y, Lovicu FJ, Overbeek PA. (1998) Lens-specific expression of transforming growth factor beta1 in transgenic mice causes anterior subcapsular cataracts. *J. Clin. Invest.* 101:625–34.
65. Shim K, Minowada G, Coling DE, Martin GR. (2005) Sprouty2, a mouse deafness gene, regulates cell fate decisions in the auditory sensory epithelium by antagonizing FGF signaling. *Dev. Cell.* 8:553–64.
66. Yu T, Yaguchi Y, Echevarria D, Martinez S, Basson MA. (2011) Sprouty genes prevent excessive FGF signalling in multiple cell types throughout development of the cerebellum. *Development.* 138:2957–68.
67. Zhao H, Yang Y, Rizo CM, Overbeek PA, Robinson ML. (2004) Insertion of a Pax6 consensus binding site into the alphaA-crystallin promoter acts as a lens epithelial cell enhancer in transgenic mice. *Invest. Ophthalmol. Vis. Sci.* 45:1930–9.
68. Ashery-Padan R, Marquardt T, Zhou X, Gruss P. (2000) Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes. Dev.* 14:2701–11.
69. Reneker LW, et al. (2004) Chick delta1-crystallin enhancer influences mouse alphaA-crystallin promoter activity in transgenic mice. *Invest. Ophthalmol. Vis. Sci.* 45:4083–90.
70. Lovicu FJ, Steven P, Saika S, McAvoy JW. (2004) Aberrant lens fiber differentiation in anterior subcapsular cataract formation: a process dependent on reduced levels of Pax6. *Invest. Ophthalmol. Vis. Sci.* 45:1946–53.
71. Zhao H, et al. (2008) Fibroblast growth factor re-

- ceptor signaling is essential for lens fiber cell differentiation. *Dev. Biol.* 318:276–88.
72. Iyengar L, Patkunanathan B, Lynch OT, McAvoy JW, Rasko JEJ, Lovicu FJ. (2006) Aqueous humour- and growth factor-induced lens cell proliferation is dependent on MAPK/ERK1/2 and Akt/PI3-K signalling. *Exp. Eye Res.* 83:667–78.
 73. Boros J, Newitt P, Wang Q, McAvoy JW, Lovicu FJ. (2006) Sef and Sprouty expression in the developing ocular lens: implications for regulating lens cell proliferation and differentiation. *Semin. Cell Dev. Biol.* 17:741–52.
 74. Lovicu FJ, McAvoy JW. (2001) FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling. *Development.* 128:5075–84.
 75. Taliana L, Evans MD, Ang S, McAvoy JW. (2006) Vitronectin is present in epithelial cells of the intact lens and promotes epithelial mesenchymal transition in lens epithelial explants. *Mol. Vis.* 12:1233–42.
 76. Wang Q, Stump R, McAvoy JW, Lovicu FJ. (2009) MAPK/ERK1/2 and PI3-kinase signalling pathways are required for vitreous-induced lens fibre cell differentiation. *Exp Eye Res.* 88:293–306.
 77. Klein OD, et al. (2008) An FGF signaling loop sustains the generation of differentiated progeny from stem cells in mouse incisors. *Development.* 135:377–85.
 78. Kuracha MR, et al. (2011) Spry1 and Spry2 are necessary for lens vesicle separation and corneal differentiation. *Invest. Ophthalmol. Vis. Sci.* 52:6887–97.
 79. Sasaki A, et al. (2003) Mammalian Sprouty suppresses Ras-independent ERK activation by binding to Raf1. *Nat. Cell Biol.* 5:427–32.
 80. Iyengar L, Wang Q, Rasko JEJ, McAvoy JW, Lovicu FJ. (2007) The duration of MAPK/ERK1/2 phosphorylation induced by FGF or ocular media determines lens cell fate. *Differentiation.* 75:662–8.
 81. Novotny GE, Pau H. (1984) Myofibroblast-like cells in human anterior capsular cataract. *Virchows Arch. A Pathol. Anat. Histopathol.* 404:393–401.
 82. Pau H, Novotny GE. (1985) Ultrastructural investigations on anterior capsular cataract. Cellular elements and their relationship to basement membrane and collagen synthesis. *Graefes Arch. Clin. Exp. Ophthalmol.* 223:41–6.
 83. Tang J, Salzman IJ, Sable MD. (2003) Traumatic cataract formation after vigorous ocular massage. *J. Cataract. Refract. Surg.* 29:1641–2.
 84. Sasaki K, et al. (1998) Early lens changes seen in patients with atopic dermatitis applying image analysis processing of Scheimpflug and specular microscopic images. *Ophthalmologica.* 212:88–94.
 85. Caporossi A, et al. (1998) Histology of anterior capsule fibrosis following phacoemulsification. *J. Cataract. Refract. Surg.* 24:1343–6.
 86. Nishi O, Nishi K, Sakka Y, Sakuraba T, Maeda S. (1991) Intercapsular cataract surgery with lens epithelial cell removal. Part IV: capsular fibrosis induced by poly(methyl methacrylate). *J. Cataract Refract. Surg.* 17:471–7.
 87. Wormstone IM. (2002) Posterior capsule opacification: a cell biological perspective. *Exp. Eye Res.* 74:337–47.
 88. Gordon-Thomson C, de Iongh RU, Hales AM, Chamberlain CG, McAvoy JW. (1998) Differential cataractogenic potency of TGF-beta1, -beta2, and -beta3 and their expression in the postnatal rat eye. *Invest. Ophthalmol. Vis. Sci.* 39:1399–409.
 89. Seomun Y, Kim J, Lee EH, Joo CK. (2001) Overexpression of matrix metalloproteinase-2 mediates phenotypic transformation of lens epithelial cells. *Biochem. J.* 358:41–8.
 90. Cerra A, Mansfield KJ, Chamberlain CG. (2003) Exacerbation of TGF-beta-induced cataract by FGF-2 in cultured rat lenses. *Mol. Vis.* 9:689–700.
 91. Banh A, et al. (2006) Lens-specific expression of TGF-beta induces anterior subcapsular cataract formation in the absence of Smad3. *Invest. Ophthalmol. Vis. Sci.* 47:3450–60.
 92. Derynck R, Zhang Y. (2003) Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature.* 425:577–84.
 93. Xu J, Lamouille S, Derynck R. (2009) TGF-beta-induced epithelial to mesenchymal transition. *Cell Res.* 19:156–72.
 94. Mulder KM, Morris SL. (1992) Activation of p21ras by transforming growth factor beta in epithelial cells. *J. Biol. Chem.* 267:5029–31.
 95. Hartsough MT, et al. (1996) Altered transforming growth factor signaling in epithelial cells when ras activation is blocked. *J Biol. Chem.* 271:22368–75.
 96. Hayashida T, Poncelet AC, Hubchak SC, Schnaper HW. (1999) TGF-beta1 activates MAP kinase in human mesangial cells: a possible role in collagen expression. *Kidney Int.* 56:1710–20.
 97. Sato M, et al. (2002) Role of p38 MAPK in transforming growth factor beta stimulation of collagen production by scleroderma and healthy dermal fibroblasts. *J. Invest. Dermatol.* 118:704–11.
 98. Jiang W, et al. (2010) Role of cross-talk between the Smad2 and MAPK pathways in TGF-beta1-induced collagen IV expression in mesangial cells. *Int J Mol. Med.* 26:571–6.
 99. Mulder KM. (2000) Role of Ras and Mapks in TGFbeta signaling. *Cytokine Growth Factor Rev.* 11:23–35.
 100. Hayashida T, Decaestecker M, Schnaper HW. (2003) Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-beta-dependent responses in human mesangial cells. *FASEB J.* 17:1576–8.
 101. Roelen BA, et al. (2003) Phosphorylation of threonine 276 in Smad4 is involved in transforming growth factor-beta-induced nuclear accumulation. *Am. J. Physiol. Cell Physiol.* 285:823–30.
 102. Horiguchi K, et al. (2009) Role of Ras signaling in the induction of Snail by transforming growth factor-beta. *J. Biol. Chem.* 284:245–53.
 103. Kretzschmar M, Doody J, Timokhina I, Massagué J. (1999) A mechanism of repression of TGFbeta/Smad signaling by oncogenic Ras. *Genes Dev.* 13:804–16.
 104. Dawes LJ, Sleeman MA, Anderson IK, Reddan JR, Wormstone IM. (2009) TGFbeta/Smad4-dependent and -independent regulation of human lens epithelial cells. *Invest. Ophthalmol. Vis. Sci.* 50:5318–27.
 105. Xie L, et al. (2004) Activation of the Erk pathway is required for TGF-beta1-induced EMT in vitro. *Neoplasia.* 6:603–10.
 106. Wormstone I, Eldred J, Dawes L, Reddan J, Wang L. (2008) TGFbeta signalling through Smad-independent mechanisms in human lens cells [abstract]. *Acta Ophthalmol.* 86 Suppl s243: [no pagination given online]. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/j.1755-3768.2008.4144.x/abstract>