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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 (TGFβ), is involved in the epithelial-to-mesenchymal transition (EMT) leading to the pathogenesis of a diverse range of fibrotic diseases. In lens, aberrant TGFβ 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 TGFβ-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 TGFβ-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 TGFβ 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 TGFβ-induced EMT.

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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 gastrulation 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 would 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 (TGFβ) has been the most intensively studied regulator of EMTs in both normal and pathological conditions (19). Given the increasing complexity of TGFβ-mediated signaling, our understanding of the required mechanisms leading to EMT is becoming more complex. The canonical TGFβ-Smad signaling cascade can crosstalk 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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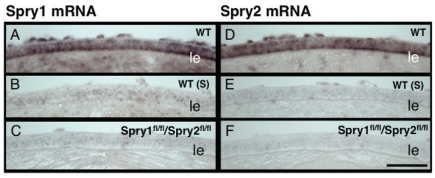

Figure 1. Expression of Spry1 and Spry2 transcripts in the neonatal mouse lens. Representative micrographs of *in situ* hybridization labeling for Spry1 mRNA (A–C) or Spry2 mRNA (D–F) using antisense (A,C,D,F) or sense (B,E) digoxygenin-labeled riboprobes, on WT lenses (A,B,D,E) or transgenic lenses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

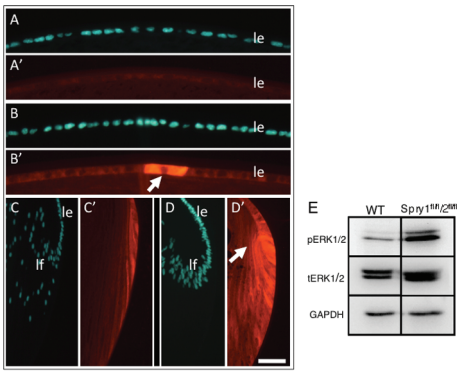

Figure 2. Spry-deficient lenses display increased immunolabeling for ERK1/2 (pERK1/2) phosphorylation in the postnatal mouse lens. Representative anterior (A, A′, B, B′) and equatorial (C, C′, D, D′) sections of postnatal-d-21 WT (A, A′, C, C′) and Spry-deficient (B, B′, D, D′) lenses using MLR10-Cre, 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 (Spry$^{fl/fl}$/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 μm; C, C′, D, D′ = 65 μm.

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 TGFβ-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 TGFβ, with Spry transcripts downregulated in response to TGFβ 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 TGFβ-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 TGFβ signaling in the eye results in an EMT that bears a morphologic and molecular resemblance to some forms of human cataract, including anterior subcapsular cataract (ASC) and posterior capsular opacification (PCO; 56). Reports of increased levels of TGFβ in many eye diseases (57), together with other studies *in vitro* and *in vivo*, have shown that TGFβ-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 TGFβ 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 TGFβ-mediated signaling, in turn blocking EMT and cataract formation. Although the specific signaling pathways and

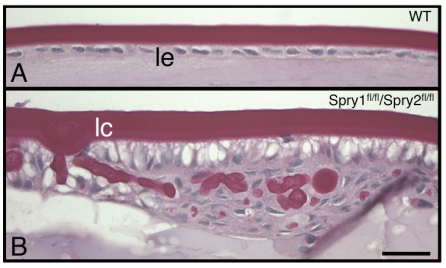

Figure 3. Spry-deficient murine lenses develop anterior subcapsular plaques. Representative histological sections of postnatal-d-80 WT mouse lenses (A) or mouse lenses deficient for all alleles of Spry1 and Spry2 (Spry$^{fl/fl}$/Spry2$^{fl/fl}$, B) using MLR10-Cre, stained with periodic acid Schiff reagent. 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 μm.

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 αA-crystallin promoter, fused to a chick δ1-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 TGFβ lines (64,70), to overexpress Spry1 in this specific background. As previously reported, for genotyping, PCR primers specific to the δ1-crystallin enhancer element (69) or the SV40 polyadenylation region (64) were used to genotype our Spry1- or TGFβ-transgenic lines, respectively. All transgenic animals characterized (that is, Cre, Spry1, TGFβ) were hemizygous for their respective transgene.

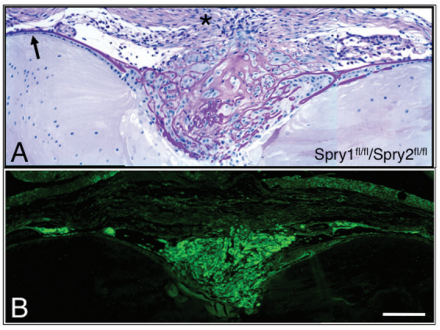

Figure 4. Spry-deficient murine lenses develop anterior subcapsular plaques with evidence of an EMT. Representative histological sections of postnatal-d-26 mouse lenses deficient for all alleles of Spry1 and Spry2 (Spry$^{fl/fl}$/Spry2$^{fl/fl}$) using Le-Cre, stained with PAS reagent (A) or immunolabeled 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 μm.

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-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 bisbenzimide (Hoechst dye; Sigma 33342) at 2 μg/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

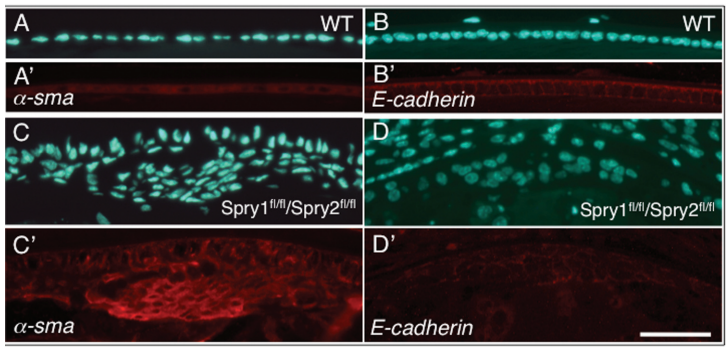

Figure 5. Epithelial cells from Spry-deficient murine lenses undergo an EMT, resulting in anterior subcapsular fibrotic plaques. Representative sections of postnatal-d-80 (A, A′, C, C′) or postnatal-d-10 (B, B′, D, D′) WT mouse lenses (A, A′, B, B′) or lenses deficient for all alleles of Spry1 and Spry2 ($Spry^{fl/fl}/Spry2^{fl/fl}$) 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 μm.

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 μg 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 TGFβ-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% CO_2. 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 vulgaris lectin (30 μg/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),

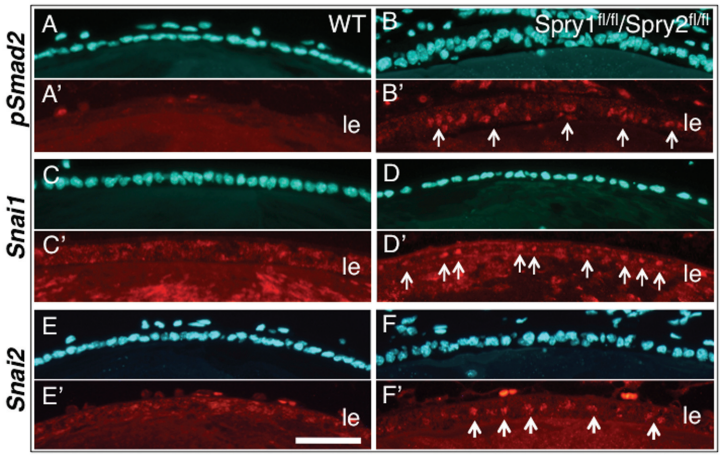

Figure 6. Epithelial cells from Spry-deficient murine lenses display TGFβ-like signaling. Representative sections of neonatal WT mouse lenses (A, A′, C, C′, E, E′) or lenses deficient for all alleles of *Spry1* and *Spry2* (Spry$^{fl/fl}$/Spry2$^{fl/fl}$) by using Le-Cre (B, B′, D, D′, F, F′), immunolabelled 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 TGFβ-mediated signaling molecules (B′, D′, F′; arrows), prior to cell multilayering to form anterior subcapsular plaques. Scale bar, 50 μm.

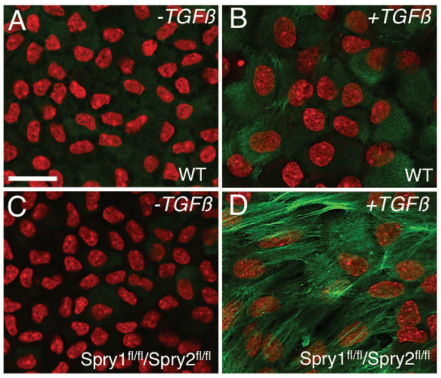

Figure 7. Spry-deficient lens epithelial cells are more responsive to TGFβ-induced EMT. Epithelial explants prepared from postnatal-d-15 WT murine lenses (A, B) or lenses deficient for all alleles of Spry1 and Spry2 (Spry$^{fl/fl}$/Spry2$^{fl/fl}$) by using MLR10-Cre (C, D) were exposed to either no TGFβ (A, C) or 200 pg/mL TGFβ (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 TGFβ, with greater immunolabeling for α-sma (D) compared with WT lens cells (B) that express lower endogenous Spry levels. Scale bar, 20 μm.

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). Quantification 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 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

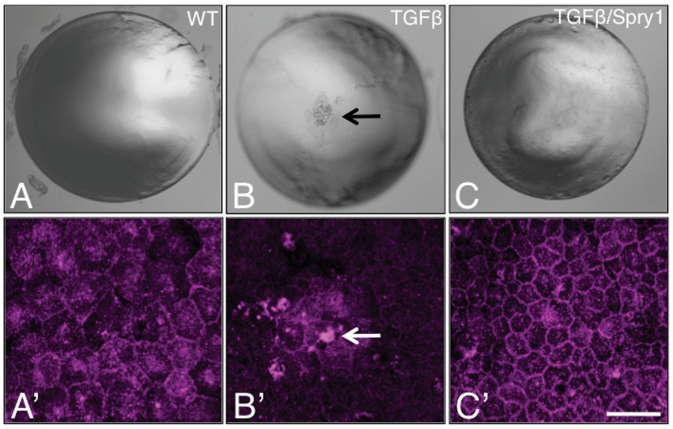

Figure 8. Spry can prevent TGFβ-induced ASC formation. Representative phase-contrast images of whole lenses (A–C) or epifluorescent images of lens epithelial whole mounts, with cell membranes stained with TRITC-conjugated lectin (A'–C'), from postnatal-d-15 lenses from either (A, A') WT mice, (B, B') transgenic mice overexpressing the mature form of human TGFβ1 specifically to the lens (TGFβ)or (C, C') transgenic mice overexpressing TGFβ1 as well as Spry1 specifically to the lens (TGFβ/Spry1). In contrast to the transparent lens (A) and uniform monolayer of lens epithelia (A') from WT mice, lenses from transgenic mice overexpressing TGFβ1 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 TGFβ1 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 μm.

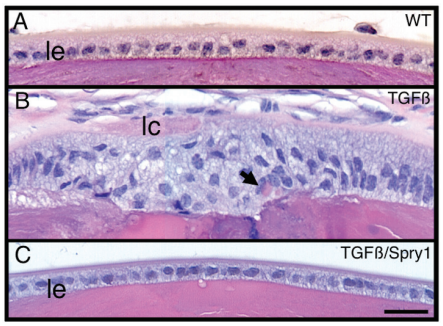

Figure 9. Spry can prevent TGFβ-induced anterior subcapsular plaque formation. Representative histological sections of postnatal-d-10 (A) WT mouse lenses and transgenic mouse lenses (B) overexpressing TGFβ1 (TGFβ) or (C) both TGFβ1 and Spry1 (TGFβ/Spry1), stained withPAS reagent. The anterior monolayer of central lens epithelial cells (le) of WT mice (A) becomes multilayered with elevated levels of TGFβ1 (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 TGFβ1 prevents the formation of this anterior subcapsular plaque formation (C), with the lens epithelial cells remaining as a monolayer (C). Scale bar, 25 μm.

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 TGFβ Signaling

Given that elevated TGFβ 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 TGFβ signaling (nuclear localization of pSmad2, Snai1 and Snai2) in neonatal Spry-deficient lenses, prior to disruption of the lens 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 TGFβ signaling.

To determine whether epithelial cells from these Spry-deficient lenses were more sensitive to the effects of TGFβ, 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 TGFβ. After a 24-h culture period, TGFβ-treated lens epithelial cells from WT mice underwent

an EMT and were immunoreactive for α-sma (Figure 7B), compared with cells not treated with TGFβ (Figure 7A). Under the same conditions, lens epithelial cells in explants deficient for Spry demonstrated a more pronounced TGFβ-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 TGFβ, cultured Spry-deficient lens epithelial cells appeared similar to cells from WT mice (Figure 7C).

Spry Can Block TGFβ-Induced ASC Formation

Because a deficiency in Spry in lens cells makes them more susceptible to the effects of TGFβ *in vitro* and leads to aberrant TGFβ 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 TGFβ-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 TGFβ 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 TGFβ, 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-

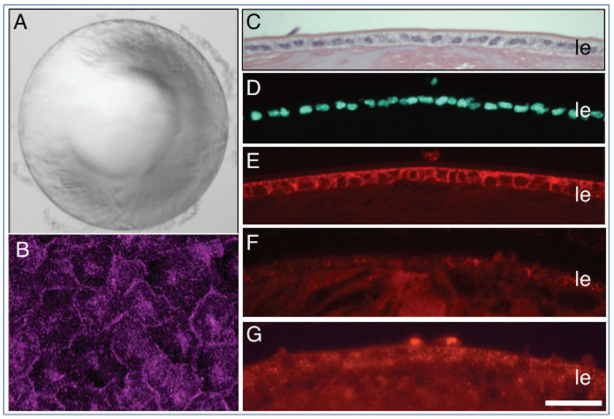

Figure 10. Representative images from postnatal-d-15 (A, B) or neonatal (C–G) Spry1-overexpressing transgenic mice depicting phase-contrast microscopy of a whole transparent lens (A), epifluorescence of an intact lens epithelial wholemount stained with TRITC-conjugated lectin (B), PAS-staining (C) or 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 μm; C-G = 35 μm.

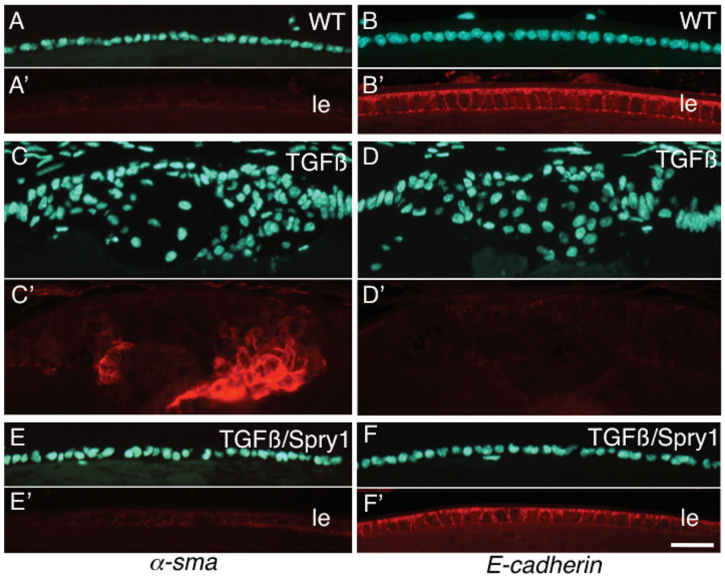

Figure 11. Spry can prevent TGFβ-induced EMT leading to anterior subcapsular cataract formation. Representative sections of postnatal-d-10 WT mouse lenses (A, A', B, B') or transgenic mouse lenses overexpressing TGFβ1 (C, C', D, D'; TGFβ) or both TGFβ1 and Spry1 (E, E', F, F'; TGFβ/Spry1), immunolabelled 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 TGFβ 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 TGFβ (D') as the cells multilayer and undergo an EMT (D). In lens epithelial cells (le) of transgenic mice overexpressing TGFβ 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 μm.

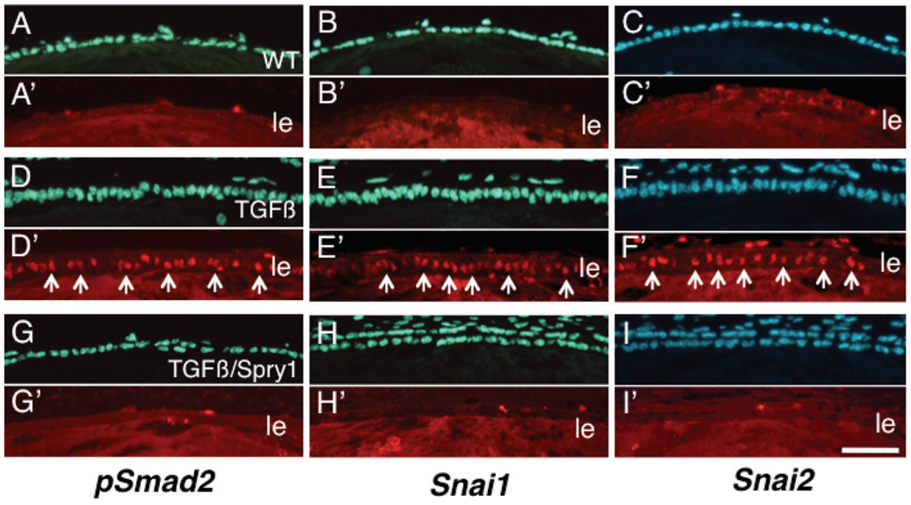

Figure 12. Spry can block TGFβ-mediated signaling in lens cells. Representative sections of neonatal WT mouse lenses (A, A′, B, B′, C, C′), or transgenic mouse lenses overexpressing TGFβ1 (D, D′, E, E′, F, F′; TGFβ) or both TGFβ1 and Spry1 (G, G′, H, H′, I, I′; TGFβ/Spry1), immunolabelled for either 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 TGFβ demonstrate a clear nuclear label for all three of these TGFβ-mediated signaling molecules (D′, E′, F′; arrows). In lens epithelial cells (le) of transgenic mice overexpressing TGFβ 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 μm.

tive of TGFβ overexpression. In mice up to 21 d old, cataracts were evident only in lenses overexpressing TGFβ (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 TGFβ (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 TGFβ there was evident multilayering of lens epithelial cells, accompanied by aberrant PAS-stained ECM and lens capsule remodeling (Figure 9B), all hallmarks of TGFβ-induced EMT and ASC progression. In contrast, lenses overexpressing Spry1, in the presence of TGFβ, 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 maintenance of the monolayer of lens epithelia (Figures 10A–C).

Spry Can Block TGFβ-Induced EMT

Given that the morphological changes accompanying TGFβ-induced ASC formation were blocked in the presence of Spry1, we examined whether the molecular markers associated with TGFβ-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 TGFβ 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 TGFβ 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 TGFβ-Signaling in the Lens

We have shown Spry to block EMT and subsequent ASC formation when highly expressed in transgenic mice overexpressing TGFβ specifically in the lens. To determine whether this block to EMT and ASC formation is associated with a block to TGFβ signaling, we again assayed for markers of TGFβ signaling (nuclear localization of pSmad2, Snai1 and Snai2) in neonatal lenses exposed to TGFβ, 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 TGFβ 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 TGFβ, consistent with a block to TGFβ-induced EMT (Figure 11) and ASC formation (Figures 8 and 9), there is also a block to TGFβ 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 TGFβ-Induced EMT in Lens Epithelial Cells

Our *in vivo* studies have shown Spry expression results in reduced TGFβ 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 TGFβ, and not through some other indirect pathway *in situ*, we compared the effects of increasing concentrations of TGFβ 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 TGFβ2 (Figure 13B) when compared with untreated cells (Figure 13A). With higher concentrations of TGFβ2, 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 TGFβ 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 TGFβ, similar to lens cells not treated with TGFβ (Figure 13E). However, application of markedly higher doses of TGFβ (up to 200 pg/mL) on the Spry-overexpressing lens cells was sufficient to overcome the antagonistic effects of Spry on TGFβ activity, because cells underwent an EMT, with strong reactivity for α-sma evident (Figure 13H).

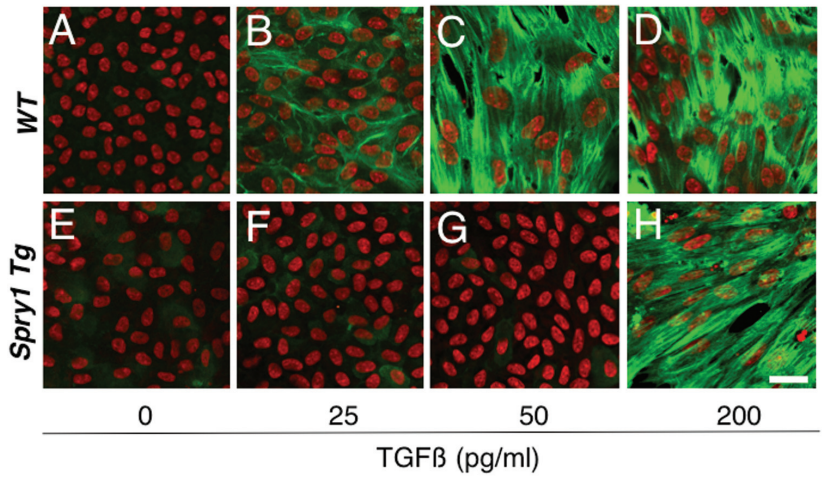

Figure 13. Spry specifically blocks TGFβ-activity in lens epithelial explants. Epithelial explants prepared from postnatal-d-15 WT murine lenses (A–D) or transgenic mouse lenses overexpressing Spry1 (E-H) were exposed to no TGFβ (A, E), 25 pg/mL TGFβ2 (B, F), 50 pg/mL TGFβ2 (C, G) or 200 pg/mL TGFβ2 (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 TGFβ (B), and the degree of this EMT was progressively more pronounced with increasing concentration of TGFβ (C, D). In contrast, lens epithelial cells overexpressing Spry1 were more resistant to the effects of TGFβ, with little to no response with either 25 pg/mL (F) or 50 pg/mL (G) TGFβ. At much higher concentrations of TGFβ (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 μm.

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 TGFβ,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, resulting 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

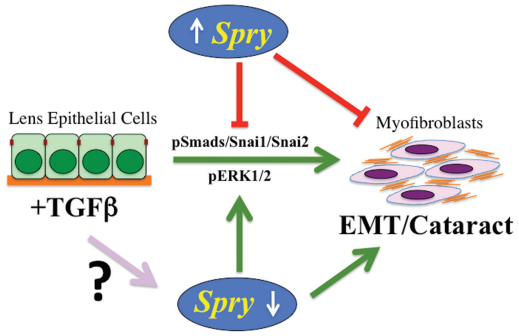

Figure 14. Influence and interaction of TGFβ signaling and Spry on the epithelial phenotype, leading to the progression or prevention of an EMT in the lens.

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 TGFβ in its pathogenesis (56,87). Similar to what we report for this study, others have shown TGFβ-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 TGFβ-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 TGFβ 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 TGFβ-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 TGFβ. Compared to WT lens epithelia, epithelial cells expressing higher levels of Spry1 were more resilient to the effects of TGFβ, neither undergoing an EMT nor accumulating α-sma. This was not the case with higher levels of TGFβ 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 TGFβ 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 TGFβ-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 TGFβ is not yet clear, but we have demonstrated that lens epithelial cells deficient for Spry are more sensitive to TGFβ compared with WT lens epithelium. This result is supported by the fact that downstream TGFβ-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 TGFβ in our transgenic mice. If we overexpress Spry in the lenses of these TGFβ-overexpressing mice, as above, we no longer see this activation and nuclear translocation of Smad2, Snai1 and Snai2; indicative of Spry influencing TGFβ-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, TGFβ has been reported to signal via Smad-independent pathways, such as RhoA, PI3-K/Akt and the MAPKs, including

ERK1/2, JNK (c-Jun NH_2-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).

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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.

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