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Smad3 Signaling Is Required for Epithelial-Mesenchymal Transition of Lens Epithelium after Injury

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Lens epithelial cells undergo epithelial-mesenchymal transition (EMT) after injury as in cataract extraction, leading to fibrosis of the lens capsule. Fibrosis of the anterior capsule can be modeled in the mouse by capsular injury in the lens, which results in EMT of the lens epithelium and subsequent deposition of extracellular matrix without contamination of other cell types from outside the lens. We have previously shown that signaling via Smad3, a key signal-transducing element downstream of transforming growth factor (TGF)-β and activin receptors, is activated in lens epithelial cells by 12 hours after injury and that this Smad3 activation is blocked by administration of a TGF-β2-neutralizing antibody in mice. We now show that EMT of primary lens epithelial cells *in vitro* depends on TGF-β expression and that injury-induced EMT *in vivo* depends, more specifically, on signaling via Smad3. Loss of Smad3 in mice blocks both morphological changes of lens epithelium to a mesenchymal phenotype and expression of the EMT markers *snail*, α-smooth muscle actin, lumican, and type I collagen in response to injury *in vivo* or to exposure to exogenous TGF-β in organ culture. The results suggest that blocking the Smad3 pathway might be beneficial in inhibiting capsular fibrosis after injury and/or surgery. *(Am J Pathol 2004, 164:651–663)*

Certain cells have an inherent plasticity such that their morphology and phenotype can be modulated by various growth factors and extracellular stimuli. As an example, the ability of an epithelial cell to change its morphology and its transcriptional program to that characteristic of a mesenchymal cell, or so-called epithelial-mesenchymal transition (EMT), is important not only in development, but also in wound healing, fibrosis, and invasion and metastasis of tumor cells.[1–3]

Although lens epithelial cells are derived from surface ectoderm, they express vimentin[4] as well as the epithelial surface marker, N-cadherin.[5] Transdifferentiation of these cells into elongated mesenchymal-like cells involves transcriptional reprogramming as evidenced by expression of type I collagen and α-smooth muscle actin (α-SMA).[6–9] This well-established EMT is readily observed after injury *in vivo* or in cell culture. EMT in these cells *in vivo* results in fibrosis and/or contraction of the capsular tissue.[6,9] Similar injury-induced EMT is observed after cataract surgery, although in this operation the entire lens content is removed and the cells migrate to the posterior capsular surface resulting in fibrosis of the posterior capsule as well as the residual anterior capsule.[9–12] The resultant fibrosis, referred to as postoperative capsular opacification, can impair patients' vision. Animal lenses are exceptionally suitable for detailed analysis of EMT *in vivo*, because the lens contains only one epithelial cell type and there is little chance of contamination with other cells after injury. A puncture wound in the anterior capsule of a mouse lens is sealed by fibrotic tissue, containing α-SMA-positive fibroblastic-like lens cells.[10–12]

Growth factors, including especially transforming growth factor (TGF)-β, orchestrate the EMT of various epithelial tissues in response to injury.[1–3,13,14] TGF-β2 is a likely mediator of EMT in lens epithelial cells *in vivo*, because it is expressed at much higher levels than the other TGF-β isoforms in the aqueous humor that bathes the lens tissue,[15] as well as in the vitreous.[16] TGF-β2 also up-regulates α-SMA in lens epithelial cells *in vitro* and in organ culture.[17] TGF-β signals through a pair of transmembrane receptor serine-threonine kinases and down-

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stream mediators called Smad proteins. Receptor-activated Smad proteins, Smad2 and Smad3, are phosphorylated directly by the TβRI receptor kinase, partner with the common mediator, Smad4, and translocate to the nucleus where they play a prominent role in activation of TGF-β-dependent gene targets.[14,18] Despite the importance of this pathway in mediating transcriptional effects of TGF-β on cells,[19,20] its role in mediating EMT is controversial.[21–29] Such studies are based on use of a relatively limited number of cell lines *in vitro*, and none have addressed the role of Smad signaling in EMT *in vivo*, in processes such as response to injury. We have previously reported that activation of Smad3/4 signaling in lens epithelial cells after capsular injury was blocked by an injection of neutralizing antibody to TGF-β2 in mice,[10] indicating that injury-induced Smad3/4 signaling is likely to be mediated by TGF-β2. A similar nuclear translocation of Smad3/4 is observed after cataract surgery in humans lens epithelial cells.[11] These findings led us to hypothesize that injury-induced EMT of lens epithelium is likely initiated by activation of TGF-β/Smad3 signaling.

In the present study, we have directly addressed the role of TGF-β/Smad3 signaling in EMT of lens epithelial cells both *in vitro* and *in vivo*. We use a TGF-β-neutralizing antibody, to show that endogenous TGF-β is involved in the initiation of EMT in primary porcine lens epithelial cells *in vitro*. Most importantly, we have used Smad3$^{ex8/ex8}$ [knockout (KO)] mice[30] to show that EMT of lens epithelium after injury *in vivo* is completely blocked in the absence of Smad3, consistent with the absence of expression of EMT markers including, *snail*,[31] lumican, α-SMA, and collagen seen in eyes of Smad3$^{+/+}$[wild-type (WT)] littermates. Together these results suggest that Smad3 is required for injury-induced EMT in lens epithelium and that inhibition of this pathway might be desirable clinically to prevent capsular opacification, which can be a complication of cataract surgery.[7]

Materials and Methods

All of the experimental procedures were approved by the Animal Care and Use Committee of the National Cancer Institute, National Institutes of Health, Bethesda, MD, and that of Wakayama Medical University, Wakayama, Japan, and conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

EMT of Primary Culture of Porcine Lens Epithelial Cells

Anterior lens capsules with an epithelial layer, obtained from a pig eye, were put in a 30-mm collagen-coated plastic culture dish to allow the epithelial cells to outgrow. After reaching confluence, the cells were trypsinized, suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and seeded on fibronectin-coated chamber slides (Falcon; Becton Dickinson, Lincoln Park, NJ). Twenty-four hours later fresh culture medium supplemented with either 20 μg/ml of monoclonal pan-specific TGF-β-neutralizing antibody (R&D Systems, Minneapolis, MN) or bovine serum albumin was added and the cells were incubated for an additional 36 hours. Cells were then fixed with 4% paraformaldehyde in 0.1 mol/L of phosphate buffer, processed for immunocytochemistry for α-SMA as described below, and mounted in balsam. The percentage of α-SMA-positive cells was determined by scoring expression in 100 cells taken from three independent areas.

For Western blotting of α-SMA, passage two primary lens epithelial cells were grown until subconfluent in two 25-cm^2 fibronectin-coated culture bottles (Iwaki Glass, Tokyo, Japan) in culture medium supplemented with 10% fetal calf serum. They were then further incubated in serum-free Dulbecco's modified Eagle's medium with either 20 μg/ml of monoclonal pan-specific TGF-β-neutralizing antibody (R&D Systems) or nonimmune IgG at the same concentration for an additional 72 hours. The cells were scraped, collected, and immediately mixed with 2× sample buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA), and blocked in 5% skim milk in phosphate-buffered saline (PBS). After incubation with primary antibodies against α-SMA (1:500 dilution in PBS, clone 1A4; Neomarker, UK) and actin (1:500 dilution in PBS; Santa Cruz Biochemicals, Santa Cruz, CA) at 4°C overnight, blots were reacted with peroxidase-conjugated secondary antibodies and developed with enhanced chemiluminescence (Amersham Biosciences, Buckingshire, UK).

Subcapsular Injury in Mouse Eyes

Adult Smad3-KO and WT mice (4 to 6 weeks old; 72 KO and 72 WT mice) were anesthetized with an intraperitoneal injection of pentobarbital sodium (70 mg/kg).[30] A small incision was made in the central anterior capsule with the blade part of a 26-gauge hypodermic needle through a corneal incision in right eye after topical application of mydriatics and oxybuprocaine eyedrop as anesthetic. The left eye served as uninjured control. The depth of injury was ~300 μm or approximately one-fourth of the length of the blade part of the needle that we have reported previously leads to the formation of fibrotic tissue around the capsular break.[12] After instillation of ofloxacin ointment, the animals were allowed to heal for 6 hours to 8 weeks. Proliferating cells were labeled by an intraperitoneal injection of bromodeoxyuridine (BrdU); mice were killed 2 hours later by CO_2 asphyxia and cervical dislocation and each eye was enucleated. Each time point is represented by six mice of each genotype; eyes of each genotype (both injured and uninjured controls) were fixed and embedded in paraffin.

Lens Capsular Explant Culture

Ten-day-old WT (n = 7) and KO (n = 5) mice from two litters were killed as described above and both lenses

were enucleated. The lens capsule was carefully dissected and placed on fibronectin-coated chamber slides (Falcon, Becton Dickinson). The explants were incubated in Dulbecco's modified Eagle's medium-10% fetal calf serum for 12 days to allow the lens epithelial cells to grow out from the explanted lens. The maximum distance of outgrowth of the epithelial cell sheet from capsular specimen was measured and compared between WT and KO specimens to evaluate cell migratory activity. After fixation in 4% paraformaldehyde in 0.1 mol/L of phosphate buffer for 24 hours, the capsule was removed from the chamber and processed for immunofluorescence staining for α-SMA. For Western blotting of explanted specimens for α-SMA, lens capsules obtained from 10-day-old mice were incubated as above for either 6 (four WT and three KO specimens) or 12 (four WT and four KO specimens) days in a 12-well culture plate (Corning/Iwaki Glass, Corning, NY). The cells and explanted capsular specimens were mixed in 2× sample buffer and processed for Western blotting for α-SMA as described above.

Organ Culture of Lenses

The crystalline lens was carefully removed from enucleated eyes of adult Smad3-KO or -WT mice and processed for organ culture as previously described.[12] Three lenses were used in each culture condition. The lens was incubated in Dulbecco's modified Eagle's medium supplemented with antibiotics in the presence and absence of porcine TGF-β2 (10 ng/ml) with a medium change every 2 days. After 5 or 10 days of culture, the tissue was fixed in 2.0% paraformaldehyde as described above. Paraffin sections were processed for histology and immunohistochemistry.

Histology and Immunohistochemistry

Sections (5 μm) were deparaffinized and stained with hematoxylin and eosin alone or with polyclonal antibodies against collagen types I and V (both 1:100 dilution in PBS; Southern Biotechnology, Birmingham, AL), rabbit polyclonal anti-lumican antibody (10 μg/ml),[32] or with a mouse monoclonal anti-α-SMA antibody (1:100 dilution in PBS; NeoMarker, Fremont, CA), rabbit polyclonal antibodies against the TGF-β isoforms as previously reported,[33] or with nonimmune IgG (control). After binding of labeled secondary antibody and the color reaction with 3,3′-diaminobenzidine, sections were counterstained with methyl green and mounted in balsam. For the explant experiments, cells were processed for immunofluorescence staining for α-SMA and mounted in VectaShield with 4,6-diamidino-2-phenylindole nuclear staining (Vector Laboratories, Burlingame, CA).

In Situ *Hybridization for Snail and α-SMA mRNAs*

Digoxigenin-labeled riboprobes for mouse snail and α-SMA were prepared as previously reported using a digoxigenin labeling kit (Roche Diagnostics Corp-Boehringer Mannheim, Indianapolis, IN).[32] In brief, digoxigenin-11-UTP-labeled single-strand sense and anti-sense riboprobes were prepared from polymerase chain reaction products obtained from plasmids containing cDNA inserts for complete mouse *snail*[34] or α-SMA mRNAs. Polymerase chain reaction primers were as follows; 5′-CTGCTCTGCCTCTAGCACAC-3′ and 5′-TTAAGGGTAGCACATGTCTG-3′ for α-SMA and 5′-ACACTGGTGAGAAGCCATTC-3′ and 5′-AGTTCTATGGCTCGAAGCAG-3′ for *snail*. Paraffin sections, 5-μm thick, were subjected to the Ventana HX system of *in situ* hybridization (Ventana Medical Systems, Inc., Tucson, AZ) according to the manufacturer's protocol. In brief, paraffin sections were deparaffinized and digested with proteinase K (Ventana) at 37°C for 2 minutes. After hybridization, sections were washed three times in 0.1× standard saline citrate high-stringency solution (Ventana) at 65°C for three times. Probes were detected with alkaline phosphatase-conjugated anti-digoxigenin antibody of Fab fragments (Roche) at 37°C for 30 minutes. Sections were removed from the system and color developed in freshly prepared substrate solution nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (DIG nucleic acid detection kit). Slides were counterstained with nuclear red.

Results

EMT of Lens Epithelial Cells in Vitro *Depends on TGF-β*

Primary porcine lens epithelial cells exhibited a fibroblast-like morphology and expressed α-SMA, an established marker for EMT in lens epithelial cells,[7–9] 48 hours after culturing on fibronectin-coated chamber slides (Figure 1a). Addition of 20 μg/ml of pan-specific neutralizing antibodies to TGF-β suppressed the up-regulation of α-SMA as revealed by immunocytochemistry (Figure 1b). Whereas 88 ± 6.1% of the cells expressed α-SMA in the absence of antibody, only 11 ± 6.6% of the cells showed detectable staining after incubation with anti-TGF-β for 48 hours (Figure 1c). This was further confirmed by Western blotting of lysates of cells grown in serum-free medium for α-SMA (Figure 1d). Together these data indicate that TGF-β expressed by the lens epithelial cells stimulates cells to undergo EMT, as indicated by expression of the marker α-SMA.

Histology of Injured Lenses of Smad3-Knockout Mice

We have previously shown that lens epithelial cells *in vivo* undergo EMT by demonstrating acquisition of a fibroblastic morphology and expression of α-SMA, an established marker for EMT in this cell type, after nuclear translocation of Smads3/4 in response to capsular injury in mice.[10,12] In this study, nuclear translocation of Smad3/4 was detected within 12 hours after injury, whereas expression of α-SMA protein was not detected until 5 days.[10] To determine whether Smad3 might actually be

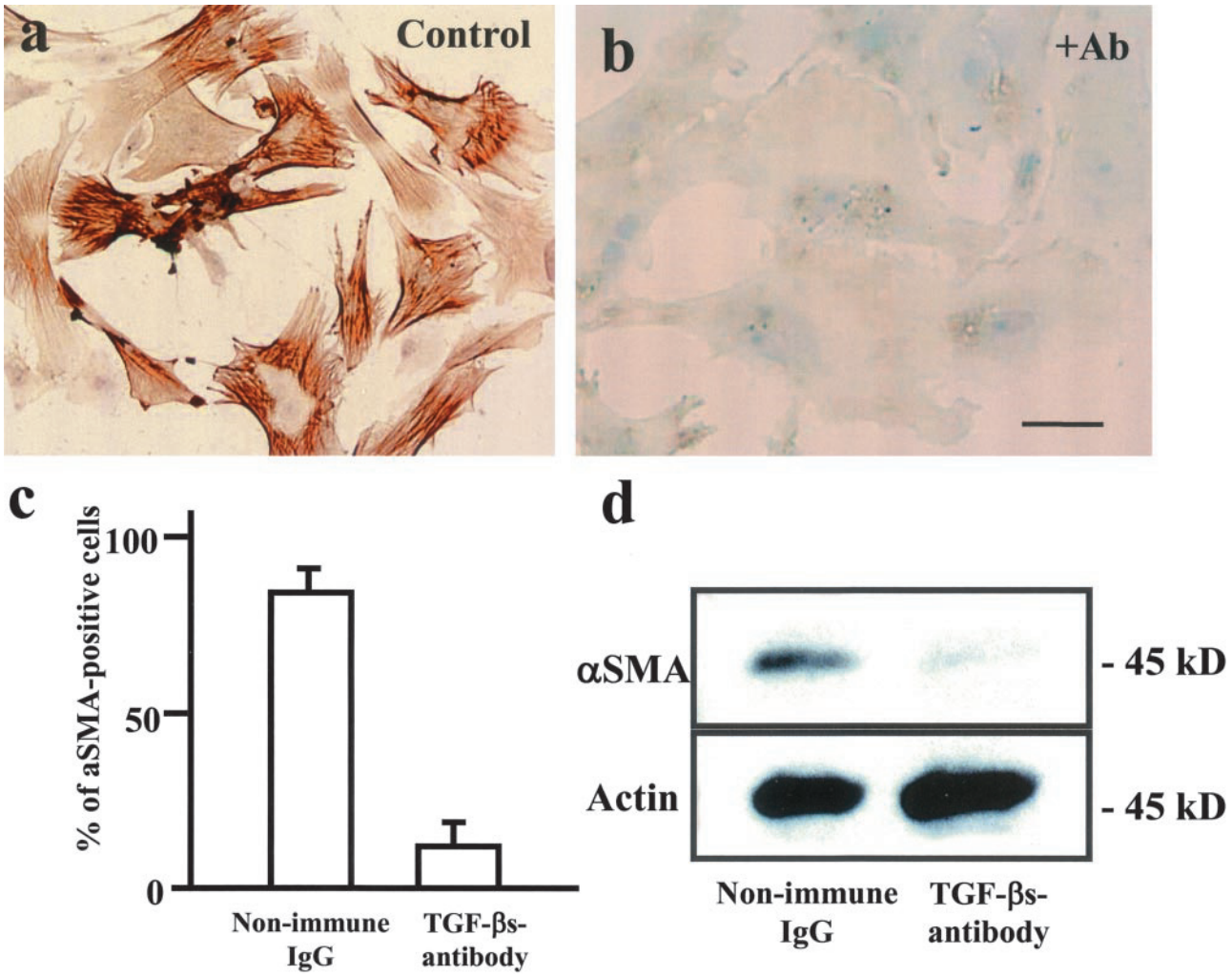

Figure 1. EMT of primary lens epithelial cells *in vitro* is dependent on endogenous TGF-β. EMT in primary porcine lens epithelial cells as evidenced by staining for α-SMA at 48 hours of culture (**a**) is blocked by addition of a neutralizing pan-specific antibody against TGF-β (20 μg/ml) (**b**). Indirect immunostaining by diaminobenzidine color reaction methyl green counterstaining. **c:** Percentage of α-SMA-positive cells in cultures shown in **a** and **b**. **d:** Protein expression level of α-SMA as determined by Western blot analysis of lysates of porcine lens epithelial cells cultured in serum-free medium in the presence of either nonimmune IgG or anti-TGF-β-neutralizing antibody for 72 hours. Actin serves as a loading control. Scale bar, 50 μm.

required for this injury-induced EMT of lens epithelium, we injured the lenses of Smad3-null mice (KO) and littermate wild-type (WT) mice and examined the response at different times after injury ranging from 1 day to 8 weeks. Lens epithelium of uninjured eyes (Figure 2, a and b) and injured eyes of KO and WT littermate controls exhibited a similar histology for the first 3 days after injury, but striking differences were observed at later times (Figure 2; c to f). The break in the anterior capsule of WT eyes was sealed by an accumulation of multilayered lens-derived cells with a fibroblast-like morphology at 5 days after injury (Figure 2c). A similar accumulation of cells was not observed in KO eyes (Figure 2d). Instead, the cells populating the wound area retained more of an epithelial-like morphology (Figure 2, d and f) compared to the elongated, fibroblast-like cells seen in WT specimens through week 8 (Figure 2, c and e). Notably, the morphology of KO cells at week 8 was similar to the cells of a normal, uninjured, lens (Figure 2f, arrows). In contrast, lens epithelial cells of Smad3 heterozygous mice displayed a morphology similar to that of WT mice at day 5 after injury (data not shown). These histological findings strongly suggested that loss of Smad3 blocks injury-induced EMT in lens epithelial cells in mice.

Expression of Snail in Injured Lens Is Dependent on Smad3

To further document the apparent block in EMT of lens epithelium seen in KO mice after injury, we examined the expression of both early and later markers of EMT. *Snail* is a member of a family of zinc finger-containing transcriptional repressors increasingly associated with suppression of the epithelial phenotype associated with EMT[3] and shown to be an immediate-early Smad3-dependent gene target of TGF-β in fibroblasts.[35] We therefore examined whether *snail* might also be an early marker of Smad3-dependent EMT *in vivo*. Whereas *in situ* hybridization for *snail* mRNA showed undetectable expression in

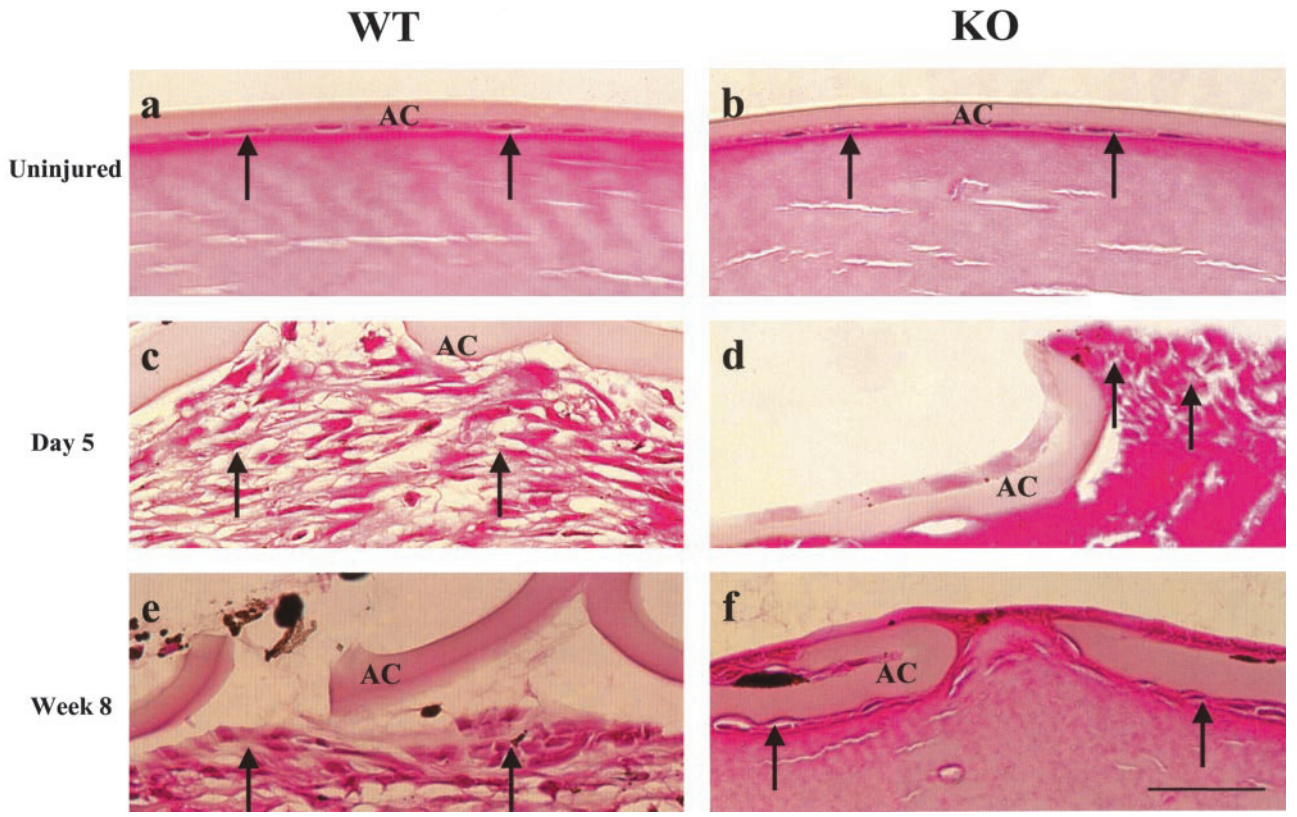

Figure 2. Histology of lens epithelial cells after capsular injury in Smad3-knockout mice. H&E-stained paraffin sections of WT (Smad3$^{+/+}$, **left**) and KO (Smad3$^{ex8/ex8}$, **right**) uninjured murine globes (**a**, **b**) or of eyes at day 5 (**c**, **d**), or week 8 (**e**, **f**) after injury. Cells in WT injured lenses are of a fibroblastic appearance, but not in KO lenses. The appearance of KO cells at week 8 is similar to that of normal lens epithelial cells (**arrows**, **f**). **Arrows** and AC indicate lens epithelial cells and anterior lens capsule, respectively. Scale bar, 50 μm.

uninjured WT or KO lenses (not shown), a signal could be seen in epithelial cells around the capsular break in WT lenses at 1 day after injury (Figure 3a, arrow) and at day 3 in the multilayer fibroblast-like cells (Figure 3c, arrows). Although fibroblast-like cells around the capsular break continued to express *snail* at later times after injury (Figure 3, e and h, arrows), epithelial cells distal to the injury site were not labeled at this (Figure 3, e and g) or at earlier times after injury (Figure 3, a and c). Lens epithelial cells in injured KO eyes never expressed *snail* mRNA (Figure 3; b, d, and f), further supporting the notion that Smad3 signaling is required for EMT of lens epithelial cells. No signal was seen with the sense riboprobe (Figure 3i).

Expression of Markers of the Later Stages of EMT in the Injured Lens Is Also Dependent on Smad3

Because the data from histological analysis and *in situ* hybridization for *snail* were suggestive of perturbed EMT in KO lens epithelium in response to injury, we examined if loss of Smad3 would also block expression of other markers characteristic of later stages of lens epithelial cell EMT such as lumican, α-SMA, and collagen type I.

Uninjured lens epithelium was negative for α-SMA protein and mRNA. WT lens epithelial cells were negative for α-SMA mRNA at day 1 after injury, but first expressed it at day 3, whereas KO epithelial cells never expressed it throughout the interval up to week 8 (data not shown). Consistent with our previous observations that lens epithelial cells undergoing EMT *in vivo* start to express α-SMA protein between days 3 and 5 after a lens capsular injury,[7–9,12] immunohistochemical staining with anti-α-SMA antibody showed that fibroblastic-like lens epithelial cells that populated the injury site in WT eyes were strongly labeled by 5 days after injury (Figure 4a) and continued to express α-SMA at 1 and 2 weeks after injury (Figure 4, c and e), returning to baseline at 8 weeks (data not shown). Immunoreactivity for α-SMA was strongest at 1 week after injury. Injured eyes of Smad3 heterozygous mice showed a morphological EMT and expression of α-SMA similar to that of WT mice, suggesting that a single allele of Smad3 was sufficient to support EMT (data not shown). In contrast, the more epithelial-like cells found in wounds in KO eyes were completely negative for α-SMA and remained so even up to 8 weeks after injury (Figure 4, b, d, and f, and data not shown).

Expression of lumican, a small, leucine-rich keratan sulfate proteoglycan, precedes up-regulation of α-SMA in lens cells during wound healing because loss of lumican delayed the expression of α-SMA in such cells after injury.[12] To gain additional insight into the role of lumican in EMT of lens epithelium and to further identify the steps

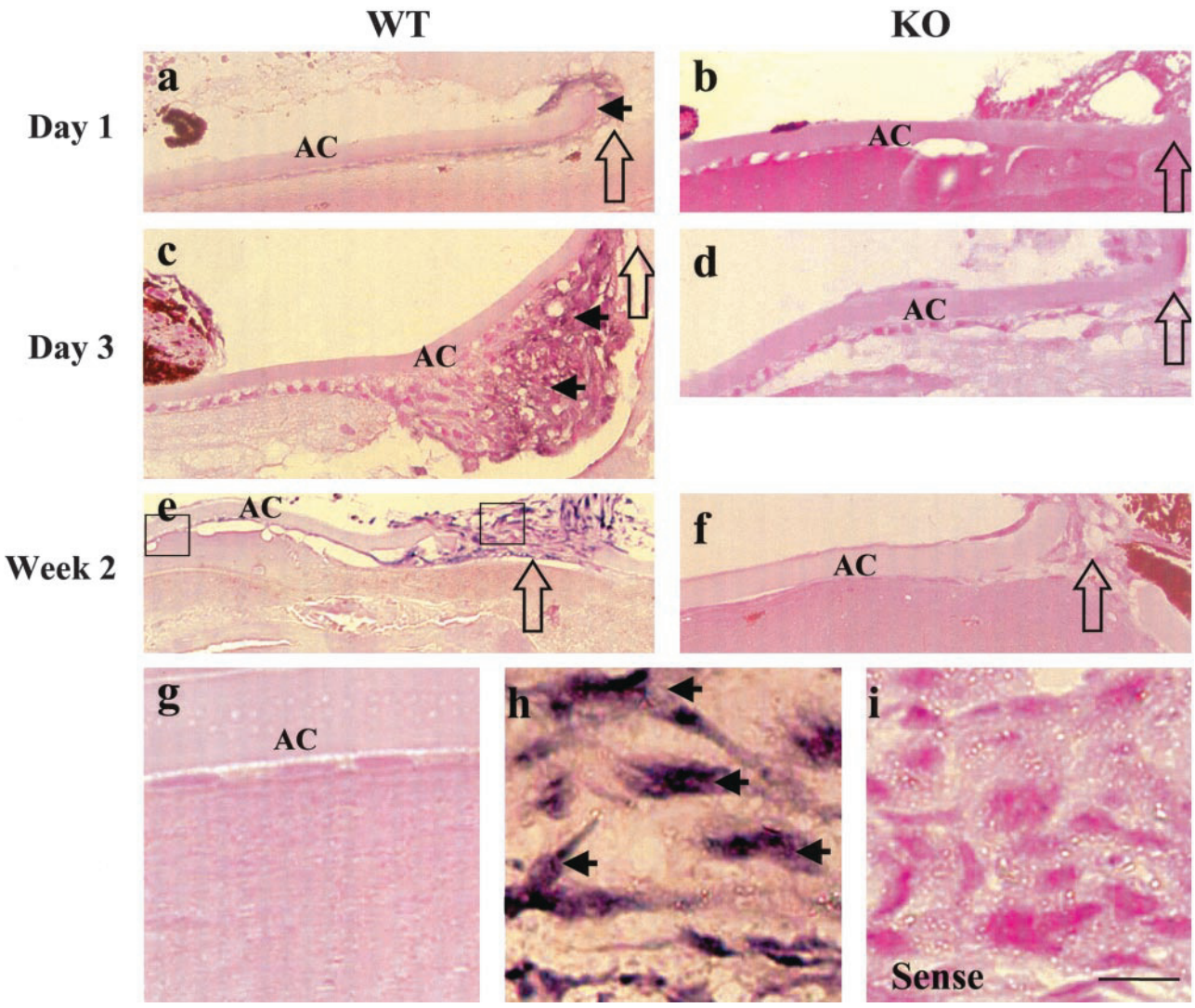

Figure 3. Smad3 is required for expression of *snail* mRNA in lens epithelial cells in response to injury. Expression of *snail* mRNA in WT (Smad3$^{+/+}$, **left**, and **g–i**) and KO (Smad3$^{ex8/ex8}$, **right**) lens epithelium at day 1 (**a**, **b**), day 3 (**c**, **d**), or week 2 after injury (**e–i**). **g** and **h:** Area high-magnification pictures of the boxed areas in **e**. **i:** Sense probe in serial section from **h**. *Snail* mRNA is detected in epithelial cells of WT injured lenses, but not in KO injured lenses. **Filled arrows** in **a**, **c**, and **h** indicate *snail* mRNA-expressing cells. **Open arrows** indicate the margin of the capsular break made by puncture injury; AC, anterior capsule. Scale bars: 50 μm (**a–f**); 12 μm (**g–i**).

dependent on Smad3, we examined the pattern of lumican expression in KO lenses after injury. Uninjured lens epithelial cells did not express lumican in either WT or KO mice (data not shown). However, whereas WT lens epithelial cells at the capsular break began to express lumican protein as early as day 1 (not shown) and cells expressing a fibroblastic morphology at 5 days after injury were positive (Figure 5a) and remained positive until 4 weeks after injury (data not shown), lens epithelial cells of KO mice were negative for lumican expression at all times examined (Figure 5b and data not shown).

As evidence that this block in EMT in lenses of KO eyes after injury also prevented subsequent fibrosis, no staining for collagen types I (Figure 5d) or V (data not shown) was evident in these eyes. In contrast, cells around the capsular break in WT eyes became weakly reactive to anti-collagen I antibody between days 2 and 3 after injury (data not shown) and remained strongly reactive up to week 8 after injury (Figure 5c). Together, these data indicate that Smad3 signaling is essential to injury-induced EMT of lens epithelial cells *in vivo*.

Late Induction of Expression of TGF-β1 after Injury Suggests a Role in Fibrosis but Not EMT

Although TGF-β2 predominates in the eye,[15,16] overexpression of TGF-β1 driven by the α-lens crystalline promoter results in EMT of the lens epithelium and formation of cataracts[36] and each of the three isoforms of TGF-β has been shown to be capable of inducing cataracterous changes in rat lenses in organ culture, albeit with different potencies.[37] To address whether TGF-β1, rather than TGF-β2, might mediate EMT of lens epithelium after injury *in vivo*, we used isoform-specific antibodies to assess their expression.[33] Uninjured lens epithelial cells in WT

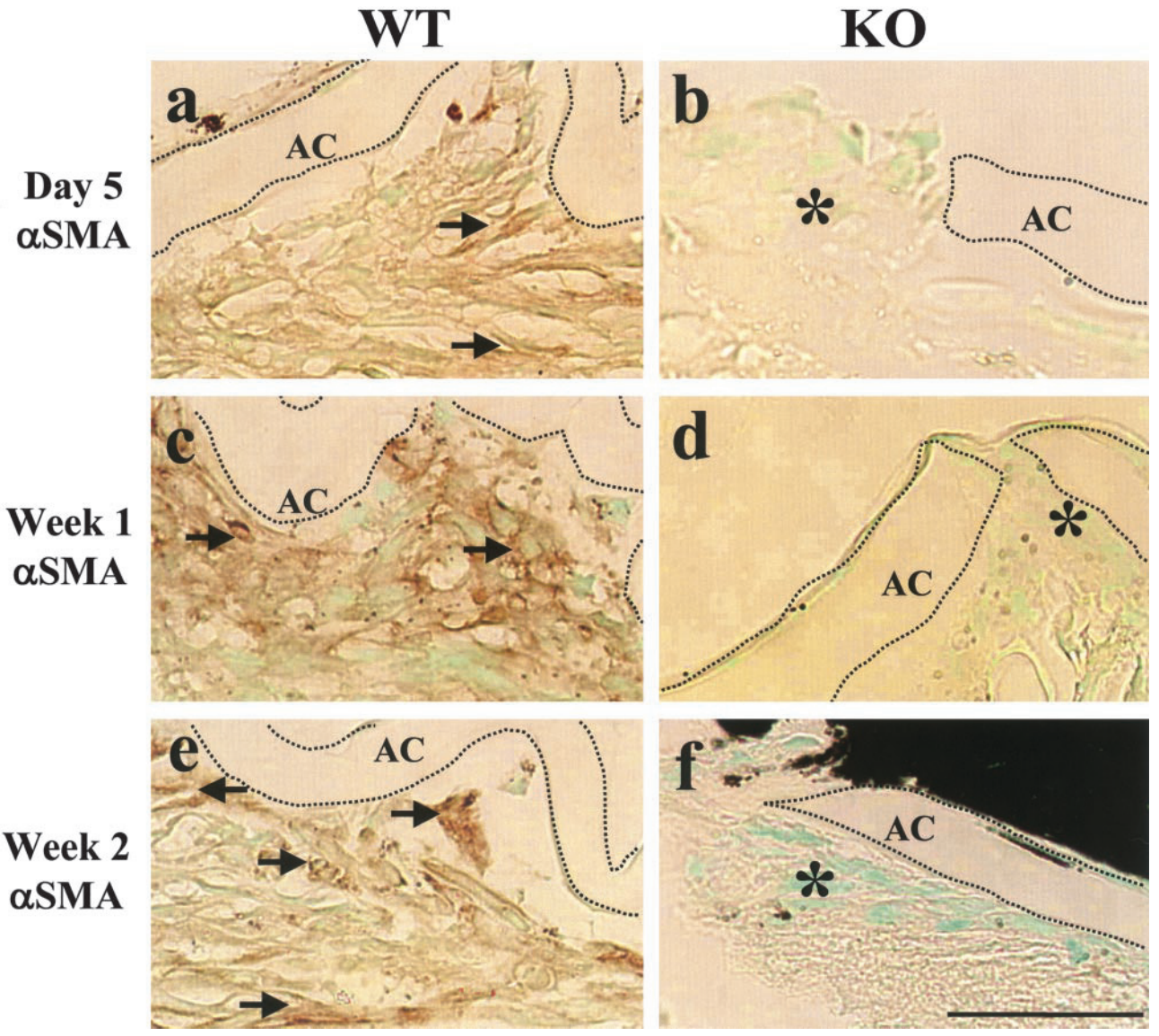

Figure 4. Smad3 is required for expression of α-SMA protein after lens injury. **a** and **b**, **c** and **d**, or **e** and **f:** Injured anterior lens tissues at day 5, week 1, or week 2, respectively. **Arrows** indicate α-SMA-expressing cells in WT mice (**a**, **c**, and **e**) and **asterisks** indicate nonexpressing cells in KO mice (**b**, **d**, and **f**). The **dotted line** with AC indicates broken anterior capsules. WT ($Smad3^{+/+}$, **left**); KO ($Smad3^{ex8/ex8}$, **right**). Scale bar, 50 μm.

and KO mice did not express detectable amounts of TGF-β1 (Figure 6, a and b). In WT mice, TGF-β1 was up-regulated in lens epithelial cells exhibiting a fibroblastic morphology at week 1 after injury (Figure 6c), increased in intensity until week 4 (Figure 6e), and then returned to basal levels at week 8 (not shown). Throughout the healing intervals examined, no up-regulation of TGF-β1 was observed in KO mice (Figure 6, d and f). These data are consistent with the observed lack of autoinduction of TGF-β1 in Smad3-null cells,[19,38] and with reduced levels of expression of TGF-β1 in skin of KO mice after irradiation.[39] The late onset of TGF-β1 expression after capsular injury and our observation that expression of α-SMA protein was unperturbed at day 5 after capsular-injury in 2-week-old *Tgf*-β1-null mice (data not shown) suggest that TGF-β1 does not play a direct role in EMT, but that it might contribute to elaboration of extracellular matrix (ECM) at later times after injury. In contrast, there was no obvious difference in expression of TGF-β2 in eyes of WT and KO mice (Figure 6; g to n). TGF-β2 was expressed in peripheral lens epithelial cells in the proliferative zone, but not in central epithelia of uninjured lenses in both WT and KO mice (Figure 6; g to j). However, at week 1 after injury, central epithelia around the capsular break became positive for expression of TGF-β2 (Figure 6, k and l), increasing by week 4 after injury (Figure 6, m and n), and this diminished by week 8 after injury in both WT and KO mice (data not shown).

Outgrowth of a Migrating Epithelial Sheet and Its Expression of α-SMA Is Perturbed by the Loss of Smad3

To further confirm that EMT of mouse lens epithelium requires Smad3 signaling, we examined EMT in explant

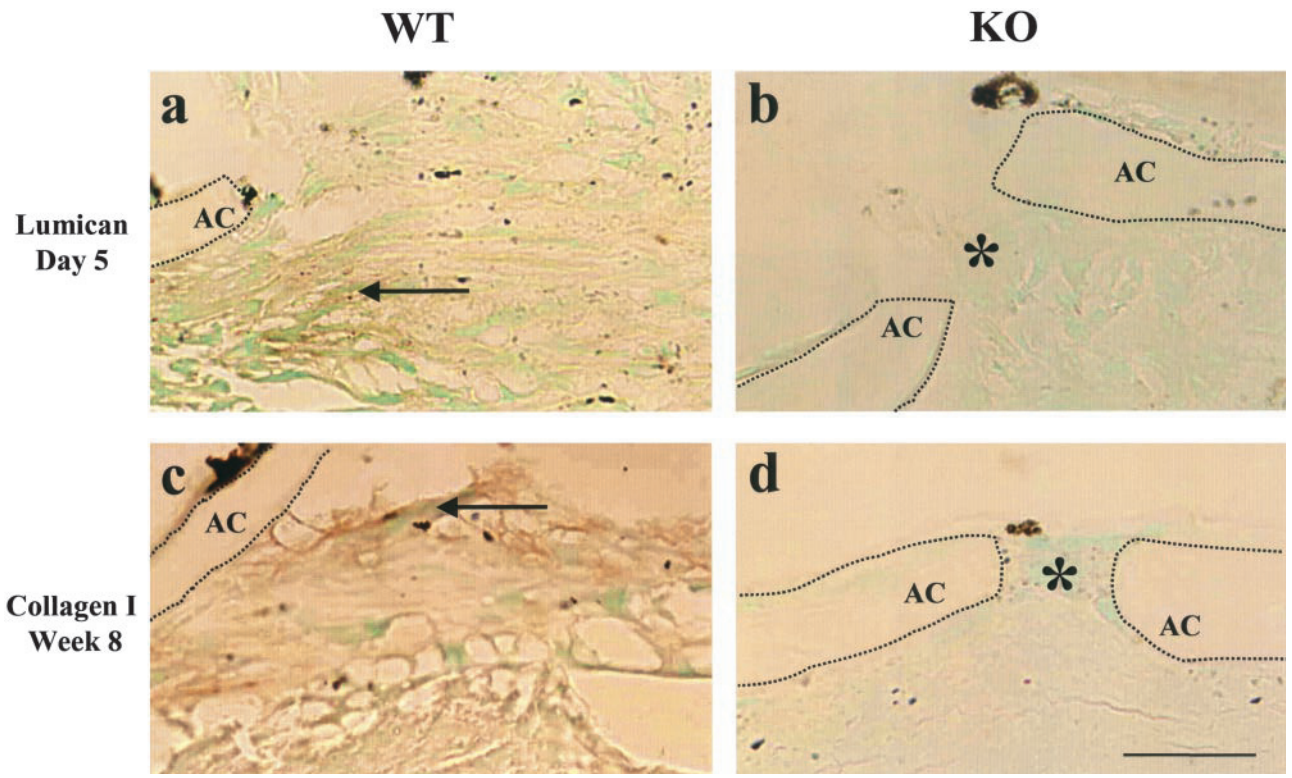

Figure 5. Extracellular matrix components, lumican and collagen type I, are expressed in epithelial cells of injured WT lenses, but not in injured KO lenses. Immunohistochemical staining for lumican (**a**, **b**) or type I collagen (**c**, **d**) at indicated times after injury in eyes of WT ($Smad3^{+/+}$, **left**) and KO ($Smad3^{ex8/ex8}$, **right**) mice. **Arrow** indicates deposition of lumican (**a**, at day 5) or collagen I (**c**, at week 8). **Asterisks** in **b** and **d** indicate cells in injured KO lenses without immunoreactivity for lumican or collagen I, respectively. The **dotted line** with AC indicates broken anterior capsules. Indirect immunostaining. Scale bar, 50 μm.

cultures of lenses of WT and KO mice. WT lens epithelial cells exhibit a more robust outgrowth from the capsular specimens than do KO cells (Figure 7; a, b, and f). Notably, WT cells located at the edge of the migrating epithelial sheet exhibited a fibroblast-like morphology and expressed α-SMA (Figure 7c), whereas no labeled cells were seen in cultures of KO specimens (Figure 7d). This selective expression of α-SMA by the WT lens epithelium was confirmed by Western blotting at days 6 and 12 of culture (Figure 7e). Similar levels of α-SMA protein were expressed by WT explant cultures at both time points examined, whereas the protein was undetectable at both time points in lysates of KO explant cultures.

TGF-β2-Mediated EMT in Lens Organ Culture Is Dependent on Smad3

We have previously reported that organ culture of mouse lens in the presence of 10 ng/ml of TGF-β2 for 10 days results in EMT and expression of α-SMA by epithelial cells beneath the intact capsule.[12] To confirm our *in vivo* results suggesting that this process is dependent on Smad3, we cultured lenses from WT and KO eyes (Figure 8) in the presence or absence of TGF-β2 (10 ng/ml) for periods up to 10 days. TGF-β2 up-regulated expression of lumican at day 5 in cultured lenses of WT but not KO (Figure 8, c and d). At this time point, there was still no evidence for morphological EMT or α-SMA expression in cultures of either genotype (Figure 8, a and b), consistent with our previous finding that lumican expression precedes morphological evidence of EMT in lens epithelium.[12] After 10 days of culture in the presence of TGF-β2, lens epithelium of WT mice consisted of a multilayer of cells of a fibroblastic morphology (Figure 8e) and expressed lumican (not shown), α-SMA (Figure 8g), and collagen type I (Figure 8i), whereas the subcapsular cells of either KO lenses cultured in the presence of TGF-β2 (Figure 8; f, h, and j) or WT lenses cultured in the absence of TGF-β2 (not shown) retained an epithelial shape and failed to express markers of EMT.

Discussion

Our data demonstrate, for the first time, that EMT of lens epithelial cells postanterior capsular injury *in vivo* is dependent on signaling through Smad3, a key signaling intermediate downstream of TGF-β and activin receptors. In the absence of Smad3, neither the earliest marker of EMT, *snail*, nor any of the other markers for later stages of EMT are expressed, including the proteoglycan lumican, α-SMA, the hallmark of myofibroblasts, or collagen type I, a major component of the pathological ECM (Figure 9). Although these data are consistent with previous data implicating TGF-β in EMT of lens epithelium,[8,10,11,40] lens epithelial cells also express activin receptors,[41] which could activate Smad3 signaling. However, based on 1) our previous demonstration that activation of Smad3/4

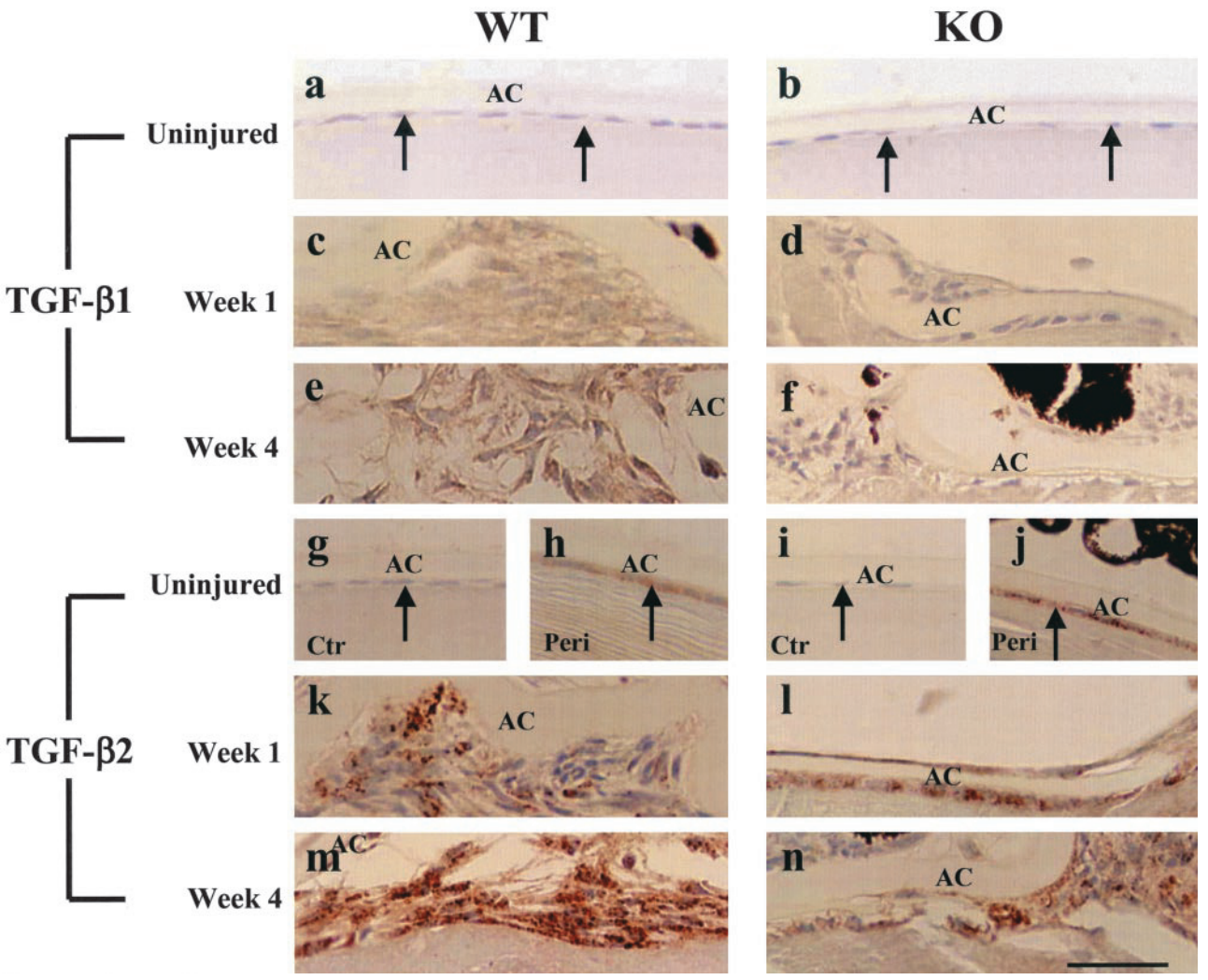

Figure 6. TGF-β1 and TGF-β2 are differentially expressed in lens epithelium after injury. Immunohistochemical staining for TGF-β1 (**a–f**) or TGF-β2 (**g–n**), at indicated times after injury in eyes of WT (Smad3$^{+/+}$, **left**) and KO (Smad3$^{ex8/ex8}$, **right**) mice. TGF-β1 protein is not detected in uninjured epithelium of WT and KO mice, but is up-regulated in WT epithelium after injury (**c**, **e**), but not in KO mice (**d**, **f**). TGF-β2 protein is observed in peripheral epithelium (**h**, **j**), but not in central epithelium (**g**, **i**) of both WT and KO mice. After injury, both mesenchymal-like cells in WT (**k**, **m**) and epithelial cells in KO (**l**, **n**) around the capsular break are labeled with anti-TGF-β2 antibody. AC, anterior capsule. Indirect immunostaining. Scale bar, 50 μm.

signaling in lens epithelium after injury was blocked by injection of an antibody to TGF-β2 in mice;[10] 2) the finding that spontaneous EMT of primary porcine lens epithelial cells is also blocked by an anti-TGF-β antibody (Figure 1); 3) the ability of TGF-β2 to induce EMT in lens organ culture (Figure 8); and 4) recent studies showing that an anti-TGF-β2-neutralizing antibody, CAT-152, is effective in preventing transdifferentiation and contraction of human capsular bags *in vitro*,[40] we propose that Smad3-dependent injury-induced EMT of lens epithelium is more likely initiated by activation of TGF-β2 than by activin. Induction of TGF-β1 in mesenchymal-like cells at later times after injury (Figure 6) is consistent with a role of this isoform in elaboration of ECM, but not in induction of EMT of lens epithelium.

Others have shown that aqueous and vitreous contain inhibitors of TGF-β, such as α2-macroglobulin, which may afford protection from unwanted EMT and fibrogenesis, both basally and after injury.[42] Additionally, it has been suggested that antibody therapies directed against TGF-β, such as CAT-152, may be able to prevent capsular opacification after cataract surgery.[40] The present studies demonstrating the central role of Smad3 in both EMT of lens epithelium and, by inference from previous studies, in the elaboration of collagens and other extracellular matrix proteins by cells expressing a mesenchymal phenotype[20,38] (Figure 9), now suggest that Smad3 might be another target for design of novel therapeutics.

EMT of cardiac endothelial cells is required for formation of the endocardial cushions in the atrioventricular canal of the developing heart.[43,44] Unlike the EMT of lens epithelium described here, this TGF-β2-dependent EMT is independent of Smad3, because heart development is normal in Smad3-null mice.[30] Rather EMT of cardiac endothelial cells is dependent on expression of the type III receptor (TβRIII) and expression of *slug*, which, like *snail*, represses expression of E-cadherin.[43,45] Similarly, TGF-β3-dependent EMT of medial edge epithelial cells, criti-

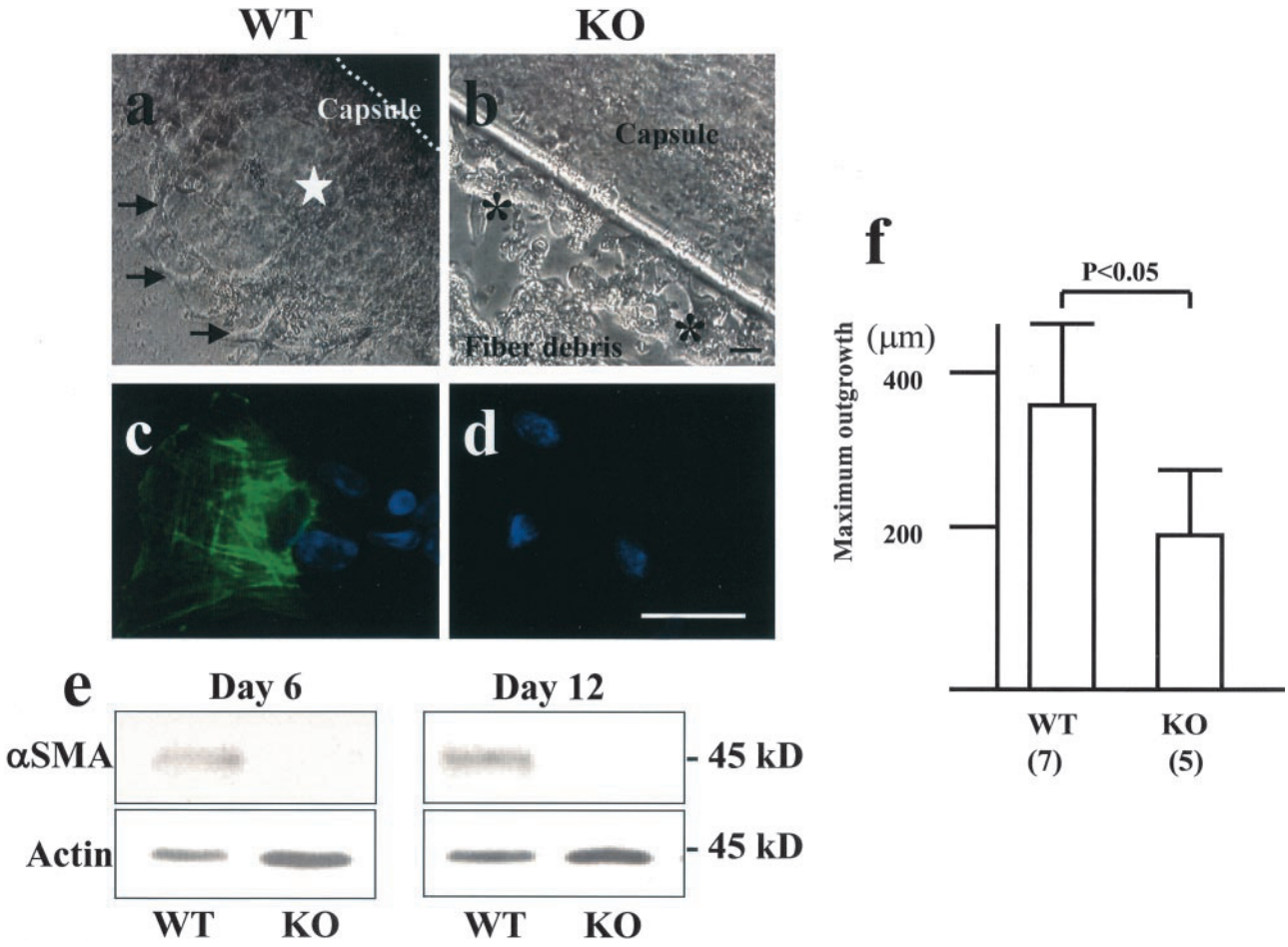

Figure 7. Smad3 is required for expression of α-SMA in outgrowths of mouse lens epithelial cells. Smad3$^{+/+}$ (WT) lens epithelial cells (**a**, **asterisk**) migrate out of capsular specimens placed in chamber slides, whereas the outgrowth of Smad3$^{-/-}$ (KO) cells (**b**, **asterisks**) is comparatively less. WT cells located at the edge of the migrating epithelial sheet (**a**, **arrows**) exhibited more of a fibroblast-like morphology compared to KO cells. Immunofluorescence staining for α-SMA identified a small number of WT cells located at the migrating edge (**c**), whereas no labeled cells were seen in cultures of KO specimens (**d**). Western blotting also showed expression of α-SMA in WT, but not KO cells at days 6 and 12 of culture (**e**). **f:** The mean value of the maximal length of cell outgrowth in each specimen with the number of specimens in each genotype is shown in parentheses. Capsule, lens capsular explant; **dotted line**, margin of the capsule; fiber debris, lens fiber contamination. Scale bar, 50 μm.

cal in fusion of the palatal shelves later in development, also occurs independently of Smad3 and correlates with expression of TβRIII and phosphorylation of Smad2.[46,47]

Several studies of EMT of epithelial cell lines in culture have also suggested that the process is independent of Smad3 and that other pathways including phosphatidylinositol 3-kinase, RhoA, and MAPK pathways are involved.[24–28] However, recent studies using a mutant TβRI unable to bind or activate Smad2/3 but still competent to signal through MAPK pathways, clearly show that Smad activation is also required in certain cells, suggesting that the Smad pathway is necessary but possibly not sufficient to effect EMT of these cell lines driven by TGF-β *in vitro*.[29,48] Based on our demonstration that EMT of lens epithelium *in vivo* is blocked in the absence of Smad3, we hypothesize that signaling through this pathway is required for the early stages of the injury-dependent multistage transition of a lens epithelial cell to a mesenchymal phenotype, but possibly no longer necessary in a subset of established cell lines that may already have transited initial Smad3-dependent steps required for induction of EMT.[24,25,28] Supporting this argument, outgrowths of lens epithelial cells from KO mice do not express α-SMA, a marker of EMT, even though it has been shown that Smad3 is not essential for induction of α-SMA by TGF-β in cultured dermal fibroblasts.[49] Similarly, in cultured hepatic stellate cells, an inhibitor of the Smad pathway, Smad7, blocks the formation of cytoskeletal fibers immunoreactive for α-SMA, without changing the level of α-SMA protein, suggesting that TGF-β/Smad signaling is required for the assembly of α-SMA in the cytoskeleton, but not for its synthesis.[50] Taken together, these data support our arguments that TGF-β/Smad3 signaling is likely required at a very early point in the process of EMT, before the step in which α-SMA is induced.

Although the entire sequence of molecular events involved in Smad3-dependent EMT of lens epithelial cells is still not known, we have been able to characterize some of the early steps in the process. Our unpublished data show that *snail*, a zinc finger transcription factor that has been strongly linked to EMT,[31,51] is up-regulated in α-TN4, a SV40-transformed mouse lens epithelial cell line, as early as 30 minutes after TGF-β addition, and before up-regulation of α-SMA (Yoo J, Saika S, and Rob-

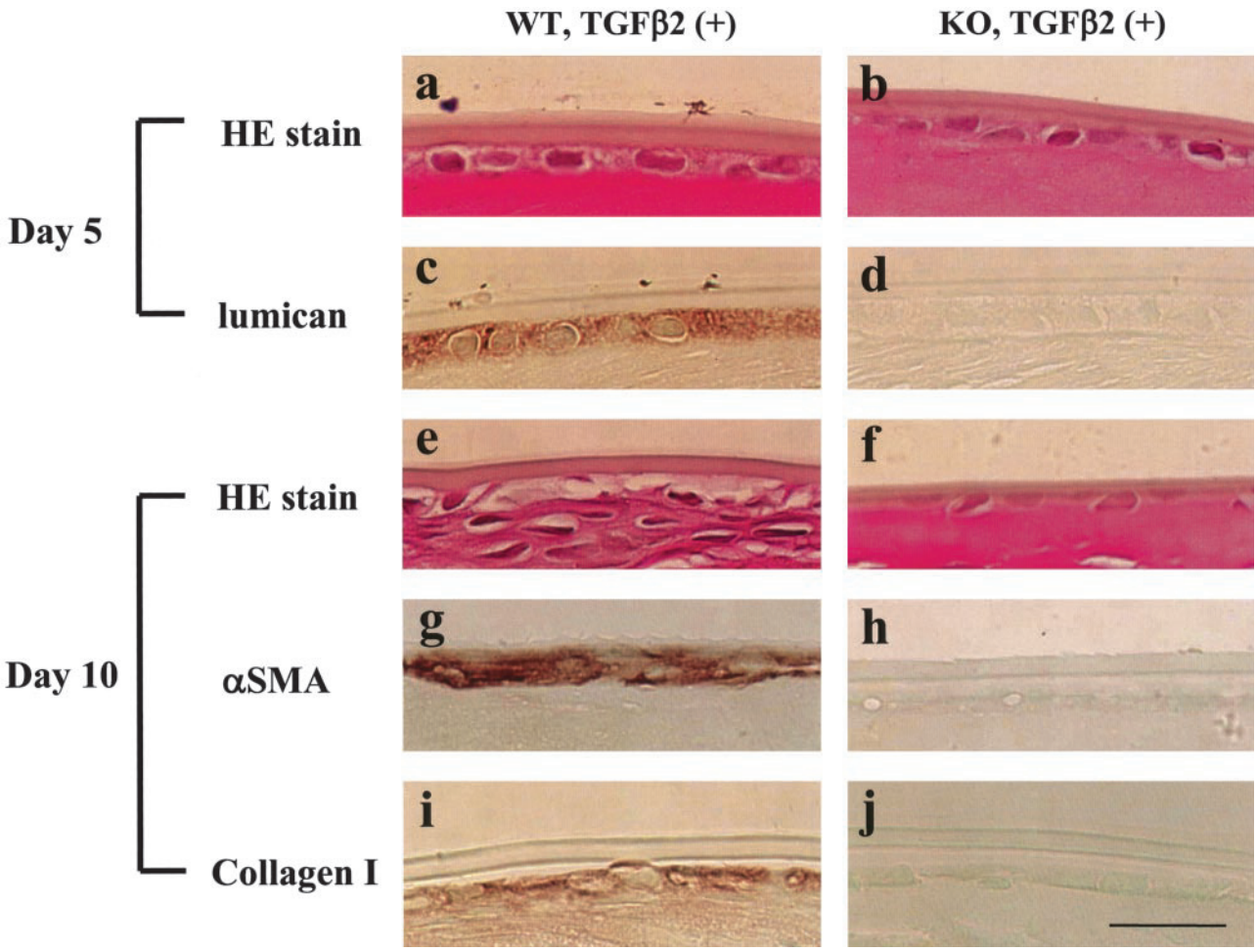

Figure 8. Induction of EMT by TGF-β2 in organ-cultured lenses requires Smad3. Lenses were cultured in serum-free Dulbecco's modified Eagle's medium supplemented with antibiotics in the presence or absence of 10 ng/ml of TGF-β2 as indicated. Sections were stained with H&E (**a**, **b**, **e**, **f**), antibodies to lumican (**c**, **d**), antibodies to α-SMA (**g**, **h**), or antibodies to type I collagen (**i**, **j**) at the indicated times as described in Materials and Methods. Lumican is expressed at day 5 and α-SMA and collagen I are expressed at day 10 in WT lenses, but not in KO lenses. WT (Smad3$^{+/+}$) lenses (**left**); KO (Smad3$^{ex8/ex8}$) lenses (**right**). Scale bar, 50 μm.

erts AB, unpublished data). Although our present data cannot address a putative requirement for *snail* for EMT, we do show that it is expressed in lens epithelial cells at the edge of the capsular break 1 day after injury, before the expression of any other markers of EMT. KO lens epithelia neither underwent EMT nor expressed *snail*, consistent with the finding that *snail* is a Smad3-dependent immediate-early gene target of TGF-β.[35] Ectopic expression of *snail* in epithelial cell lines is sufficient to induce EMT and expression of mesenchymal markers,[52] suggesting a model in which it acts as a master switch controlling the subsequent transcriptional changes. Although the *snail* homologue, *slug*, was basally expressed in α-TN4 cells (Yoo J, Saika S, and Roberts AB, unpublished data), whether it, or SIP1, another TGF-β-inducible, Smad-interacting zinc-finger protein involved in transcriptional suppression of E-cadherin expression, might also be involved in EMT of lens epithelium *in vivo* is not known at the present time.[51,53]

Previous studies have shown that many ECM molecules, in addition to performing structural roles, can also facilitate the conversion of cells to α-SMA-positive myo-

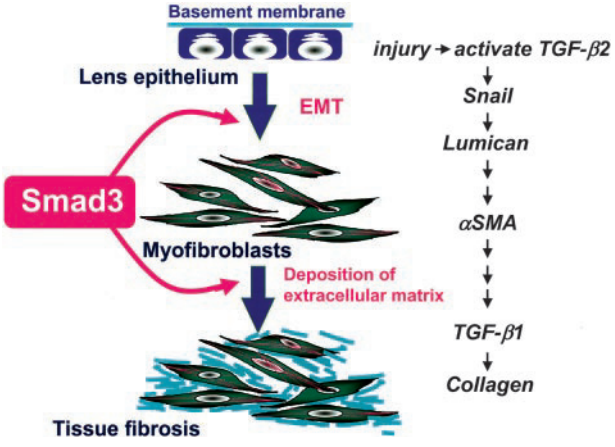

Figure 9. Smad3 is necessary for both EMT of lens epithelial cells and for subsequent elaboration of ECM proteins by myofibroblasts. Data suggest that injury-induced EMT of lens epithelial cells is initiated by activation of TGF-β2 and mediated by Smad3-dependent expression of the early marker, *snail*, followed by expression of lumican to enhance EMT of lens epithelium, and finally markers of the myofibroblast, α-SMA, and of the fibrotic phenotype, collagen I. Although loss of Smad3 blocks the process at the level of EMT, previous studies strongly implicate Smad3 in elaboration of ECM by mesenchymal cells.[20]

fibroblasts under pathological conditions.[54–56] For example, in cultured fibroblasts, fibronectin EIIIA enhances and vitronectin suppresses α-SMA expression.[57,58] The requirement of β1-integrin expression for EMT in NMuMg cells[28] and lens epithelial cells[6] again underscores the importance of ECM signaling in EMT. We have recently reported that lumican, a core protein of keratan sulfate proteoglycan,[32] is transiently expressed in healing lens epithelial cells after a puncture injury and that loss of lumican by gene targeting results in a significant delay in EMT of lens epithelial cells.[12] In the present study, we show that loss of Smad3 blocks the injury-related induction of lumican expression in lens epithelial cells. Although this loss of lumican expression may contribute secondarily to the block of EMT in the Smad3-null lens epithelium, it is unlikely to be a primary target because its loss results in a delay, but not a block of EMT.[12]

We have previously proposed that a putative inhibitor of the Smad3 pathway might promote more rapid closure of cutaneous wounds.[38] However, the wounding response in skin involves many different cell types including keratinocytes, fibroblasts, and inflammatory cells, each of which is affected in cell-specific ways by modulation of this signaling pathway. Thus the decreased expression of collagen and certain other matrix proteins in Smad3-null wounds may be not be desirable in certain situations in which wound strength might be critical. However, in the crystalline lens, the lens epithelial cell is the predominant cell lineage. Because lens epithelium is known to undergo pathological EMT after traumatic injury, as in cataract surgery and implantation of an artificial lens,[8,10,11,40] and because this EMT can lead to production of ECM and to opacification and contraction of the capsule containing the artificial lens,[7] there may be therapeutic benefit to inhibition of EMT, as proposed for antibodies to TGF-β2.[40]

Our findings implicating Smad3 signaling in EMT of lens epithelium may have broader significance based on our preliminary results using a model of retinal detachment, which show that Smad3 is also required for EMT of retinal pigment epithelium in mice. In proliferative vitreoretinopathy, which is the most common cause of failure in retinal reattachment surgery, EMT of retinal pigment epithelial cells can lead to fibrosis and to traction detachment of the retina.[16] Together, these results suggest that suppression of EMT in ocular cells by interfering with Smad3 signaling may have clinical application in treatment of these and other eye disorders.

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