Role of Epithelial–Mesenchymal Transition in Repair of the Lacrimal Gland after Experimentally Induced Injury

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PURPOSE. Ongoing studies demonstrate that the murine lacrimal gland is capable of repair after experimentally induced injury. It was recently reported that repair of the lacrimal gland involved the mobilization of mesenchymal stem cells (MSCs). These cells expressed the type VI intermediate filament protein nestin whose expression was upregulated during the repair phase. The aim of the present study was to investigate the roles of vimentin, a type III intermediate filament protein and a marker of epithelial–mesenchymal transition (EMT) in repair of the lacrimal gland.

METHODS. Injury was induced by direct injection of interleukin (IL)-1 into the exorbital lacrimal gland. MSCs were prepared from injured glands using tissue explants. Expression of vimentin and the transcription factor Snai1, a master regulator of EMT, was determined by RT-PCR, Western blotting analysis, and immunofluorescence.

RESULTS. These data show that vimentin expression, at both the mRNA and the protein levels, was upregulated during the repair phase (2–3 days postinjury) and returned to the control level when repair ended. Temporal expression of Snai1 mirrored that of vimentin and was localized in cell nuclei. Cultured MSCs isolated from injured lacrimal glands expressed Snai1 and vimentin alongside nestin and alpha smooth muscle actin (another biomarker of EMT). There was a strong positive correlation between Snai1 expression and vimentin expression.

CONCLUSIONS. It was found that EMT is induced during repair of the lacrimal gland to generate MSCs to initiate repair, and that mesenchymal–epithelial transition is then activated to form acinar and ductal epithelial cells. (*Invest Ophthalmol Vis Sci*. 2012;53:126–135) DOI:10.1167/iovs.11-7893

Epithelial–mesenchymal transition (EMT) plays major roles in tissue remodeling during embryogenesis and helps epithelial cells acquire migratory and/or invasive properties.¹ During EMT, epithelial cells lose cell–cell attachment and polarity, and epithelial-specific markers undergo cytoskeletal remodel-

ing and gain a mesenchymal phenotype.^{2–5} Downregulation of E-cadherin gene expression, an adherens junction protein, is crucial for initiation of EMT and the associated loss of cell polarity.^{2,3,5} EMT has been recently categorized into three types: type 1 EMT occurs during embryogenesis, type 2 EMT occurs during tissue repair/regeneration, and type 3 EMT occurs during tumor invasion and metastases.

The role of EMT in tissue repair/regeneration is well described. Several studies, including some that used genetic lineage-tracing methods, have shown that human pancreatic β -cells undergo EMT, before redifferentiating into insulin-producing cells.^{6–10} Similarly, EMT has been shown to occur in several other tissues, including mammary glands, liver, kidney, and lung.^{11–17} Of interest to the studies reported herein, it was shown that induction of EMT generates cells with mesenchymal stemlike properties.^{12,18,19}

Another biomarker of EMT is the expression of type III intermediate filament protein vimentin, which is normally expressed in cells of mesenchymal origin such as fibroblasts, endothelial cells, and cells of the hematopoietic lineages.⁵ Vimentin expression has been described in epithelial cells involved in organogenesis, wound healing, and tumor invasion. Impaired wound healing in embryonic and adult mice lacking vimentin has been reported and shown to be due to retarded fibroblast invasion and subsequent contraction of wounds, suggesting that vimentin is important for cell motility.²⁰ An elegant study by Gilles et al.²¹ using time-lapse video microscopy reinforces this suggestion and clearly demonstrated that vimentin expression is transiently associated and is functionally involved in the migratory status of human mammary epithelial cells in an in vitro wound-healing system. Furthermore, the relationship between the level of vimentin expression and mesenchymal cell shape and motile behavior was also recently demonstrated.²² It was shown that expression of dominant-negative mutants or silencing vimentin with short hairpin (sh)RNA causes mesenchymal cells to adopt epithelial shapes.²² Conversely, it was shown that microinjection of vimentin or transfection with vimentin complementary (c)DNA causes epithelial cells to adopt mesenchymal shapes.²²

Several transcription factors, including Snai1, Snai2, ZEB1 (δ EF1), ZEB2 (SIP1), and TWIST, have been shown to induce or contribute to EMT.^{1,4,5} However, Snai1 seems to be a master regulator of EMT and acts partly by repressing expression of E-cadherin and induction of vimentin expression.^{17,21–23} Repression of Snai1 expression is usually sufficient to induce E-cadherin expression and the cells acquire an epithelial phenotype through initiation of mesenchymal–epithelial transition (MET).^{17,21–23}

The lacrimal gland is a compound tubuloacinar exocrine gland whose secretions account for the bulk of the aqueous portion of the precorneal tear film.^{24–27} It is composed of three main cell types: acinar, ductal, and myoepithelial cells.²⁴ The acinar cells, the most abundant type (>80%), are highly polarized epithelial cells and are responsible for synthesizing, stor-

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ing, and secreting proteins, along with electrolytes and water, that are crucial for the homeostasis of the corneal and conjunctival epithelial cells of the ocular surface.^{26,27} Dysregulated lacrimal gland secretion leads to symptoms of dry eye, also known as keratoconjunctivitis sicca (KCS), a condition that tops reasons for visits to ophthalmologists.²⁸ Patients with KCS can experience intense pain arising from eye irritation, gritty/scratchy feeling in the eyes, blurry vision, and light sensitivity.²⁸ Lacrimal gland dysfunction that occurs as a result of autoimmunity, such as Sjögren's syndrome or rheumatoid arthritis, accounts for an estimated 1 to 4 million patients in the United States suffering from dry eye syndromes.^{29–31} Furthermore, KCS occurring in the absence of an underlying autoimmune disease is most prevalent in people older than 50 years of age and postmenopausal women. Recent epidemiologic studies report that an estimated 25 to 30 million Americans suffer from dry eye syndromes.^{28,32–37} These numbers are expected to rise significantly in the next decades with the aging population.^{28,33} Although the mechanisms leading to inadequate lacrimal gland secretion are not fully understood, loss of the acinar cells through programmed cell death and the inability of the tissue to replace these cells are thought to play key roles.^{38–40}

In previous studies, we reported that the murine lacrimal gland is capable of repair after experimentally induced injury.^{41,42} We showed that repair involves the recruitment and activation of MSCs to the site of injury, deposition of extracellular matrix components, and generation of new acinar epithelial cells.^{41,42} We also showed that lacrimal gland MSCs express the type IV intermediate filament (IF) protein nestin and the type III IF protein vimentin. Furthermore, a report describing the isolation, propagation, and characterization of MSCs from injured lacrimal glands was recently published.⁴³ In the present studies we sought to study in more detail the role of vimentin in lacrimal gland repair and the potential involvement of EMT in this process.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM), gentamicin, penicillin-streptomycin, heat-inactivated fetal bovine serum (FBS), and a trypsin replacement solution were used (TrypLE Express; Invitrogen, Carlsbad, CA). Also used were culture-treated plates (Corning Costar 96-well microplates; Corning Life Sciences, Union City, CA) and tissue-culture chamber slides (Lab-Tek Permanox Chamber Slides; Nalge Nunc, Inc., Penfield, NY).

The following primary antibodies were used: goat polyclonal antibody against nestin (1:40; R&D Systems, Minneapolis, MN); goat polyclonal antibody against vimentin (1:300; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal antibody against α -smooth muscle actin (1:100; Abcam Inc., Cambridge, MA); two rabbit polyclonal antibodies against Snai1 (1:250 for Western blotting analysis, 1:100 for immunofluorescence; Abcam); and mouse monoclonal antibody

against β -actin (1:5000; Sigma Chemical, St. Louis, MO). Secondary antibodies (Invitrogen) were conjugated to green-fluorescent dyes (Alexa Fluor 488, Alexa Fluor 594; Invitrogen) for immunofluorescence studies or for Western blotting analysis studies (Alexa Fluor 680, Alexa Fluor 800; Invitrogen).

Animals and Treatment

Female BALB/c mice (10–12 weeks old) were purchased from a commercial supplier (Taconic, Germantown, NY). Animals were maintained in constant-temperature rooms with fixed light/dark intervals of 12 hours' length and were fed without restriction. All experiments were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Tufts Medical Center Animal Care and Use Committee. Animals were anesthetized and the exorbital lacrimal glands were left untreated (control) or were injected with rhIL-1 α (1 μ g; a generous gift from the Biological Research Branch National Cancer Institute Preclinical Repository), as previously described.^{41,42,44} Animals were euthanized at various times postinjection and the lacrimal glands removed and divided into several pieces to be used for histology, immunofluorescence, Western blotting analysis, RNA extraction for RT-PCR analysis, or explant tissue culture.

Isolation of Mesenchymal Stem Cells from Injured Lacrimal Glands

Lacrimal gland explants were prepared from injured lacrimal glands as previously described.⁴³ Explants were washed two times in DMEM containing glucose, 2 mM L-glutamine, and sodium pyruvate and supplemented with 100 μ g/mL penicillin/streptomycin, 50 μ g/mL gentamicin, and 10% FBS (complete DMEM). Lacrimal gland explants were then placed on scored and prewet 24- or 6-well tissue-culture plates, as previously described.⁴³ The explants were allowed to adhere to the plates for 3 to 4 hours before addition of 0.2 mL of complete DMEM. Cells were grown under routine culture conditions of 95% air and 5% CO₂ at 37°C and the media was replaced every 3 days. The cells were passaged by trypsinization of confluent, adherent cells with a commercial solution (TrypLE Express; Invitrogen). These cells were partially characterized and shown to be mesenchymal stem cells (MSCs) in a previous publication.⁴³

RNA Isolation and RT-PCR Analysis

Lacrimal glands were homogenized in reagent (TRIzol; Invitrogen) and total RNA was isolated according to the manufacturer's instructions. RNA was also isolated from cultured MSCs derived from IL-1-injected lacrimal glands.

Purified total RNA (20 ng) was used for reverse transcription and PCR amplification with a commercial kit (OneStep RT-PCR Kit; Qiagen Sciences, Germantown, MD) using primers specific to vimentin, Snai1, or G3PDH (Table 1) in a thermal cycler (2720 Thermal Cycler; Applied Biosystems, Foster City, CA). G3PDH primers were purchased from a commercial supplier (ReadyMade Primers; Integrated DNA Technologies, Inc., Coralville, IA). Primers for vimentin and Snai1 were designed

TABLE 1. Sequences of Primers Used for RT-PCR

Gene	Accession Number	Primer Sequence	Amplicon (bp)	T _A (deg)
Vimentin	NM_011701.4	FW: 5'-ATGCTTCTCTGGCAGTCTT-3' RV: 5'-AGCCACGCTTTCATACTGCT-3'	206	60
Snai1	NM_011427.2	FW: 5'-AAACCCACTCGGATGTGAAG-3' RV: 5'-GAAGGAGTCCTGGCAGTGAG-3'	184	60
G3PDH	NM_008084.2	FW: 5'-ACCACAGTCCATGCCATCAC-3' RV: 5'-TCCACCACCTGTTGCTGTA-3'	452	59

G3PDH, glyceraldehydes-3-phosphate dehydrogenase; FW, forward; RV, reverse; T_A, annealing temperature.

FIGURE 1. Vimentin gene expression is upregulated in injured lacrimal glands. RNA extracted from control lacrimal glands or injured glands (injected with IL-1) was used for reverse transcription and PCR amplification using primers specific to vimentin or G3PDH. Each lane represents a sample from an individual animal. Data in the plot are mean $\pm$ SEM ($n = 3-5$). *Denotes statistically significant difference from control.

according to the mispriming library (Primer3 software; <http://fokker.wi.mit.edu/primer3/>).⁴⁵

The reverse-transcription reaction was conducted at 52°C for 30 minutes followed by PCR according to the manufacturer's instructions. The cycling conditions were 5 minutes hot start at 95°C, 25 to 30 cycles of denaturation for 40 seconds at 94°C, annealing for 40 seconds at 53°C, extension for 1 minute at 72°C, and a final extension at 72°C for 10 minutes. Samples with no RNA served as the negative controls. After amplification, the products were separated by electrophoresis on a 1.5% agarose gel and visualized by UV light after ethidium bromide staining. The amplicons were sequenced (ABI 3130XL instrument with BigDye Terminator chemistry; Applied Biosystems) and confirmed that they matched vimentin, Snai1, and G3PDH genes (using Basic Local Alignment Search Tool [BLAST], National Center for Biotechnology Information).

Histopathology and Immunofluorescence

Lacrimal glands were fixed, overnight at 4°C, in 4% formaldehyde made in phosphate-buffered saline (PBS, containing in mM: 145 NaCl, 7.3 Na₂HPO₄, and 2.7 NaH₂PO₄ at pH 7.2). Paraffin sections of the lacrimal gland (6 μ m) were deparaffinized and rehydrated using graded alcohols. For histopathology experiments, paraffin sections of the lacrimal gland were processed for hematoxylin and eosin staining.

For immunofluorescence experiments, the slides were first subjected to microwave pretreatment (20 minutes) with antigen retrieval solution (Dako, Glostrup, Denmark). After three washes in PBS, non-specific binding sites were blocked for 30 minutes using 10% donkey serum diluted in PBS. For immunofluorescence experiments with cultured cells, the cells were grown on 8-well chamber slides and fixed in 4% formaldehyde for 15 minutes at room temperature. After three washes in PBS, the cells were permeabilized, for 5 minutes, with 0.1% Triton made in PBS with 1% BSA. Nonspecific binding sites were then blocked for 30 minutes using 10% donkey serum and 1% BSA prepared in PBS. The slides were then incubated overnight at 4°C, with the indicated primary antibody diluted in PBS with 1% BSA. After three

washes in PBS, slides were incubated for 60 minutes at room temperature, with the appropriate secondary antibody diluted 1:100 in PBS. After three washes in PBS, coverslips were mounted with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) containing 4',6'-diamidino-2-phenylindole (DAPI) to stain cell nuclei. Sections were viewed using a microscope equipped for epi-illumination (Nikon UFXII Epi-Illuminator; Nikon Instruments, Melville, NY). Omission of the primary antibody or incubation with irrelevant immunoglobulins was performed for negative control experiments. Images were captured with a digital microscope camera (SPOT Digital Microscope Camera; Diagnostic Instruments, Inc., Sterling Heights, MI). Photomicrographs of double-labeled sections were opened using a commercial photo/image editing program (Photoshop; Adobe Creative Suite 4) and the number of total cells (DAPI stained), cells bearing each single stain, and those bearing both stains were counted by two independent investigators. The numbers were averaged and percentages of single-positive and double-positive cells were calculated.

Western Blotting Analysis

Lacrimal gland pieces were homogenized in 0.4 mL of ice-cold homogenization buffer (in mM: 30 Tris-HCl, pH 7.5, 10 EGTA, 5 EDTA, 1 dithiothreitol, and 250 sucrose, supplemented with protease inhibitors). After centrifugation, proteins in the supernatants were separated by SDS-PAGE (NuPage 4–12% Bis-Tris Gels; Invitrogen) followed by transfer onto nitrocellulose membranes (Invitrogen). The membranes were blocked for 60 minutes at room temperature or overnight at 4°C, in a blocking buffer (Odyssey; Li-Cor Biosciences, Lincoln, NE) diluted 1:1 in Tris-buffered saline (TBS, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl). The primary antibody was prepared in blocking buffer (Odyssey) diluted 1:1 in TBST (TBS plus 0.05% Tween 20) and incubated for 1 hour at room temperature or overnight at 4°C. After three washes in TBST, the appropriate secondary antibody (diluted 1:5000) was prepared in blocking buffer (Odyssey), diluted 1:1 in TBST, and the

FIGURE 2. Vimentin protein expression is upregulated in injured lacrimal glands. Control lacrimal glands (C) or injured lacrimal glands removed 1, 2, 3, 5, or 7 days post IL-1 injection were processed for SDS-PAGE and Western blotting analysis using an antibody against vimentin or β -actin (a loading control). Data in the plot are mean $\pm$ SEM ($n = 4$). *Denotes statistically significant difference from control (C).

membranes were incubated for 30 minutes at room temperature. After three washes in TBST, the membranes were scanned using an infrared imaging system (Odyssey Infrared Imaging System; Li-Cor Biosciences). Blotting for the structural protein β -actin was performed to control for gel loading and transfer efficiency. The immunoreactive bands acquired from the membranes were quantified using either infrared imaging software (Odyssey) or National Institutes of Health software

(ImageJ, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Data Presentation and Statistical Analysis

Where appropriate, data are expressed as mean $\pm$ SEM. The data were statistically analyzed using two-sample *t*-tests. Pearson correlations

FIGURE 3. Immunostaining for vimentin during lacrimal gland repair. Lacrimal glands removed from control or IL-1-treated animals were processed for histopathology and immunofluorescence studies using an antibody against vimentin. Cell nuclei were counterstained with DAPI. Control tissue showed very minimal basolateral vimentin staining. The amount of vimentin-positive cells increased after injury to the lacrimal gland and peaked between 2 and 3 days (when repair was at its maximum) postinjection. By day 7 post IL-1 treatment, repair is complete and vimentin staining subsided and resembled that of control glands. The results depicted in the photomicrographs were replicated at least three times on lacrimal glands from separate animals. Scale bar: 100 μ m.

were used to evaluate the linear association between *Snai1* expression and vimentin expression. Values of $P < 0.05$ were considered to be significant.

RESULTS

To begin to address the role of EMT in lacrimal gland repair, we conducted a series of experiments investigating the expression of the EMT marker vimentin during various phases of lacrimal gland repair. We first compared the level of vimentin message expression between control and injured lacrimal glands 2.5 days after IL-1 injection (to induce injury). As shown in Figure 1, vimentin mRNA expression was upregulated 4.6-fold in IL-1-injected lacrimal glands compared with control glands.

We then performed Western blotting analysis experiments to determine whether vimentin protein expression was upregulated after injury to the lacrimal glands. As shown in Figure 2, compared with control glands, the amount of vimentin protein increased, in a time-dependent manner, in IL-1-injected lacrimal glands. The post hoc *t*-test found that the 5.4-fold increase in vimentin protein expression achieved at 2 days post IL-1 injection was statistically significant compared with control tissue ($P = 0.027$).

In a third series of experiments, we used histopathology and immunohistochemistry techniques to correlate lacrimal gland repair with vimentin protein expression. As shown in Figure 3, and in accordance with our previous reports, IL-1 injection elicited an inflammatory response that was most severe at 2 days and subsided between day 5 and day 7 postinjection. Lacrimal gland repair usually starts between day 2 and day 3 after IL-1 injection.^{41,42} In parallel sections, when compared with control tissue, vimentin expression was induced in glands removed from 1-, 2-, and 3-day-injected animals (Fig. 3) and returned to control levels on day 7. Note that vimentin expression was restricted to areas undergoing tissue repair and was not detectable in the “normal”-appearing lacrimal gland lobules.

These data show that expression of vimentin, at both the message RNA level and the protein level, increased during repair of the lacrimal gland, suggesting the involvement of EMT in the repair process.

Since the upregulation of vimentin expression was reminiscent of that of nestin shown in previous studies,^{43,46} we conducted double-immunofluorescence studies on IL-1-injected lacrimal glands to determine whether these two intermediate filament proteins were expressed in the same cells. We also

FIGURE 4. Distribution of vimentin, nestin, and α SMA proteins in injured lacrimal glands. Lacrimal glands from IL-1-injected animals were removed 2.5 days post treatment and processed for immunohistochemistry. Tissue sections were stained using antibodies specific for vimentin, nestin, and α SMA. Cell nuclei were counterstained with DAPI. The staining revealed multiple populations of cells including vimentin⁺/ α SMA⁺ and vimentin⁺/nestin⁺ double-positive cells. Additionally, nestin⁺/ α SMA⁺ double-positive cells were also revealed, suggesting the possibility of a population of triple-positive cells. Arrowheads point to examples of single-labeled cells. The results depicted in the photomicrographs were replicated at least three times on lacrimal glands from separate animals. Scale bar: 25 μ m.

investigated the coexpression of vimentin with alpha smooth muscle actin (α SMA), a marker of lacrimal gland myoepithelial cells⁴⁷ and a biomarker for EMT,⁵ that we found to be occasionally coexpressed with nestin.⁴⁶ As shown in Figure 4, double-labeling experiments revealed the presence of single-labeled cells: vimentin⁺ cells ($26.6 \pm 10.1\%$), nestin⁺ cells ($7.3 \pm 2.4\%$), and α SMA⁺ cells ($10.7 \pm 2.1\%$), and double-labeled cells: vimentin⁺/nestin⁺ ($12.8 \pm 1.2\%$), vimentin⁺/ α SMA⁺ ($9.3 \pm 3.5\%$), and nestin⁺/ α SMA⁺ ($5.9 \pm 1.5\%$) double-positive cells. Note that although the same cell might harbor both proteins, if the two proteins are expressed in different cellular compartments, then the merge images in the double-

immunostaining experiment will not show a yellow color (i.e., colocalization of the two proteins; Fig. 4).

We conducted similar double-labeling experiments using MSCs isolated from IL-1-injected lacrimal glands and propagated in vitro.⁴³ We first demonstrated the expression of both vimentin message RNA as well as protein in the cultured cells at various passages (Figs. 5A, 5B). We then performed immunofluorescence experiments and, as shown in Figure 5C, the staining pattern was similar to that obtained on tissue sections: single-labeled cells: vimentin⁺ cells ($12.3 \pm 1.6\%$), nestin⁺ cells ($27.3 \pm 3.2\%$), and α SMA⁺ cells ($4.1 \pm 1.1\%$), and double-labeled cells: vimentin⁺/nestin⁺ ($44.7 \pm 5.5\%$), vimentin⁺/ α SMA⁺ ($12.3 \pm 1.6\%$), and nestin⁺/ α SMA⁺ ($5.9 \pm 1.5\%$) double-positive cells.

FIGURE 5. Expression of vimentin in cultured MSCs and injured lacrimal glands. (A) Cells isolated from IL-1-injected lacrimal glands were cultured in complete DMEM, passaged 1, 2, or 3 times and processed for RT-PCR. Each lane represents a sample from an individual animal. Expression of vimentin was detectable in all passages. (B) Isolated cells from IL-1-injected lacrimal glands from passage 1, 2, or 3 were processed for SDS-PAGE and Western blotting analysis. Each lane represents a sample from an individual animal. Similar to vimentin mRNA, vimentin protein expression was detectable in all passages. (C) Cells isolated from IL-1-injected lacrimal glands were cultured in 8-well chamber slides in complete DMEM, and processed for double immunostaining for vimentin, α SMA, and nestin. Cell nuclei were counterstained with DAPI. Similar to the immunostaining on tissue, multiple populations of cells can be seen including double-positive ones: vimentin⁺/ α SMA⁺, vimentin⁺/nestin⁺, and nestin⁺/ α SMA⁺ cells, suggesting the existence of a population of triple-positive cells. Arrowheads point to examples of single-labeled cells. The results depicted in the photomicrographs were replicated at least three times on lacrimal glands from separate animals. Scale bar: 25 μ m.

α SMA⁺ ($65.1 \pm 3.6\%$), and nestin⁺/ α SMA⁺ ($64.7 \pm 4.1\%$), double-positive cells.

These data suggest the presence of a heterogeneous population of MSCs in the lacrimal based on the expression of vimentin and nestin. They also suggest that EMT might be responsible for generating the stem cells involved in tissue repair.

The transcription factor Snai1 is a master regulator of EMT and acts partly by repressing the expression of E-cadherin while inducing the expression of vimentin.⁴⁸ Transcription of the Snai1 gene is induced when epithelial cells are forced to acquire a mesenchymal phenotype that occurs during embryogenesis and wound healing, and is also crucial for the acquisition of tumoral invasiveness, the initial step of the metastatic cascade in cancer.^{1,2,5} Based on these reports, we investigated the expression of Snai1 in cultured MSCs as well as injured

lacrimal glands using RT-PCR, Western blotting analysis, and immunohistochemistry.

In a first series of experiments, Snai1 expression was investigated using cultured MSCs. As shown in Figure 6A, Snai1 message RNA was detected in cultured MSCs from various passages. As shown in Figure 6B, Snai1 protein expression was demonstrated using immunocytochemistry, with the results showing the expected nuclear localization of this transcription factor. Using Western blotting analysis, we demonstrated the strong positive correlation between Snai1 expression and vimentin expression ($r = 0.824$, $P = 0.009$, Fig. 6C).

In the next series of experiments, we used tissue sections and lysates prepared from healing lacrimal glands to investigate Snai1 expression. As shown in Figure 7A, whereas Snai1 was undetectable in control lacrimal glands, it was expressed in tissue removed 1, 2, and 3 days postinjury. Snai1 was mainly

FIGURE 6. Expression of Snai1 in cultured MSCs. (A) Cells isolated from IL-1-injected lacrimal glands were cultured in complete DMEM, passaged 1, 2, or 3 times, and processed for RT-PCR. Each lane represents a sample from an individual animal. Expression of Snai1 mRNA was detectable in all passages. (B) Cells isolated from IL-1-injected lacrimal glands were cultured in 8-well chamber slides in complete DMEM, and processed for immunostaining for Snai1. Cell nuclei were counterstained with DAPI. Scale bar: 100 μ m. (C) MSCs were processed for SDS-PAGE and Western blotting analysis using antibodies against vimentin, Snai1, or β -actin (to control for gel loading and transfer efficiency). Each lane represents cells isolated from a different animal.

FIGURE 7. Expression of Snai1 in injured lacrimal glands. (A) Lacrimal glands removed from control or IL-1-treated animals were processed for immunofluorescence studies using an antibody against Snai1. Cell nuclei were counterstained with DAPI. Although Snai1 immunostaining was undetectable in control tissue, it was readily detected in cell nuclei of tissues removed from 1-, 2-, and 3-day injected glands. The results depicted in the photomicrographs were replicated at least three times on lacrimal glands from separate animals. Scale bar: 100 μ m. (B) Control lacrimal glands or injured lacrimal glands removed 1, 2, 3, 5, or 7 days post IL-1 injection were processed for SDS-PAGE and Western blotting analysis using antibodies against Snai1 or β -actin (to control for gel loading and transfer efficiency). Data in the plot are mean $\pm$ SEM ($n = 3$). *Denotes statistically significant difference from control (C).

localized in the cell nuclei and its expression was significantly repressed when the lacrimal gland has healed 7 days postinjury (Fig. 7A). Snai1 immunoreactivity was often associated with ductal cells (asterisks in Fig. 7A). Occasionally and especially in glands removed 3 days postinjury, Snai1 immunoreactivity could also be seen in the cytosol. It is known that Snai1 shuttles between the cytosol and the nucleus and this is regulated via phosphorylation of specific residues in Snai1.^{49–51} Phosphorylation of Snai1 leads to its export from the nucleus and degradation in the cytosol.^{49–51} Snai1 expression was confirmed using Western blotting analysis; Snai1 protein, undetectable in lysates from control lacrimal gland, was significantly upregulated 2- and 2.6-fold in lysates prepared from 1- and 2-day-injected glands, respectively, and declined by day 5 to day 7 postinjection (Fig. 7B). It is worth noting that expression of Snai1 preceded that of vimentin by approximately 1 day (Figs. 2 and 7B).

These data strongly suggest that Snai1 expression and translocation to the nucleus triggers EMT by inducing the expression of vimentin.

DISCUSSION

In the present study we present evidence showing induction of EMT during repair of the murine lacrimal gland after experimentally induced inflammation. The reexpression of vimentin during repair and its upregulation on day 2 and day 3 of the repair phase is reminiscent of that of nestin, suggesting that both proteins, along with α SMA, are important for the migration of the generated MSCs to sites of injury to initiate tissue repair. In support of such a role, we found that these proteins can be detected in the same cell both in situ as well as in vitro. Our data also imply that after EMT to generate MSCs during repair of the lacrimal gland, these cells undergo the opposite transition (i.e., mesenchymal-epithelial transition [MET]) to generate new acinar and ductal epithelial cells.

The importance of EMT and vimentin expression in tissue repair/regeneration is well described.^{1,4,8,12,14,52} In tissue culture scratch wound assays, fibroblasts isolated from vimentin null mice migrated more slowly than did their wild-type counterparts.²² Similarly, vimentin-null fibroblasts contracted collagen gel more slowly than did their wild-type counterparts.²² Several reports also showed that activation of EMT during tissue repair is responsible for the generation of stem cells or cells with stemlike properties.^{6,8,12,14,17–19,52,53} Results from the present studies suggest that EMT does occur during repair of the lacrimal gland and that it is responsible for generation of MSCs. This was supported by the finding that expression of Snai1 and vimentin, two biomarkers of EMT, was detected during the peak phase of lacrimal gland repair. Furthermore, both proteins were also expressed in MSCs isolated from injured lacrimal glands.

Snai1 appears to be the master regulator of EMT, although several other factors such as Snai2 (SLUG), ZEB1 (δ EF1), ZEB2 (SIP1), and TWIST are also known to induce or contribute to EMT.^{1,2,4,5} Repression of Snai1 expression is usually sufficient to induce E-cadherin expression and the cells acquire an epithelial phenotype through initiation of MET.^{17,21–23} Although we have not tested the expression of other transcription factors, our data suggest that Snai1 plays an important role in induction of EMT during lacrimal gland repair. Indeed, we found a temporal and spatial coordination between Snai1 expression and vimentin expression: both peaked during the repair phase (1–3 days postinjury) and declined to the control level when repair ended (7 days postinjury). Furthermore, the amount of vimentin expression in MSCs strongly and positively correlated with that of Snai1. Future experiments will test if

silencing Snai1 expression in lacrimal gland MSCs will activate MET to generate cells with an epithelial phenotype.

In our study, Snai1 immunoreactivity was often found associated with ductal cells. In other exocrine tissues, ductal cells are thought to harbor stem/progenitor cells implicated in tissue repair.^{8,54–56} Duct ligation to induce injury of the salivary glands leads to a complete loss of the acinar cells, but not the ductal cells. During the repair phase, there was increased proliferation of ductal cells or duct-associated stem cells to restore functional acinar cells.^{55–57} Similar studies performed on the pancreas suggest that ductal or ductal-associated progenitor cells are implicated in tissue repair.^{6,8,58,59} Our data suggest that Snai1 activation in the injured lacrimal gland initiates EMT in the ductal cell compartment to generate MSCs. Further experiments are needed to test this hypothesis.

In summary, our data suggest that EMT is induced during repair of the lacrimal gland to generate MSCs that migrate to the site of injury to initiate repair and that MET is then activated to form acinar and ductal epithelial cells. Investigating the molecular mechanisms controlling both EMT and MET could unravel novel strategies to restore functional lacrimal gland tissue and thus adequate production of the aqueous layer of the tear film.

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