Isolation and Propagation of Mesenchymal Stem Cells from the Lacrimal Gland

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Purpose. Previously, it was reported that the murine lacrimal gland is capable of repair after experimentally induced injury and that the number of stem/progenitor cells was increased during the repair phase (2–3 days after injury). The aim of the present study was to determine whether these cells can be isolated from the lacrimal gland and propagated in vitro.

Methods. Lacrimal gland injury was induced by injection of interleukin (IL)-1, and injection of saline vehicle served as control. Two and half days after injection, the lacrimal glands were removed and used to prepare explants or acinar cells for tissue culture. Cells derived from the explants and the acinar cells were grown in DMEM supplemented with 10% fetal bovine serum. Cells were stained for the stem cells markers, nestin, vimentin, ABCG2, and Sca-1. Cell proliferation was measured using an antibody against Ki67 or a cell-counting kit. The adipogenic capability of these cells was also tested in vitro.

Results. Results show that nestin-positive cells can be isolated from IL-1–injected, but not saline-injected, lacrimal glands. A population of nestin-positive cells was also positive for vimentin, an intermediate filament protein expressed by mesenchymal cells. In addition, cultured cells expressed two other markers of stem cells, ABCG2 and Sca-1. These cells proliferated in vitro and can be induced to form adipocytes, attesting to their mesenchymal stem cell property.

Conclusions. Murine lacrimal glands contain mesenchymal stem cells that seem to play a pivotal role in tissue repair. (*Invest Ophthalmol Vis Sci.* 2011;52:2087–2094) DOI: 10.1167/iovs.10-5686

The tear film is the interface between the external environment and the ocular surface.[1] It forms a smooth refractive surface over the corneal surface, lubricates the eyelids, and maintains the optimal extracellular environment for the epithelial cells of the cornea and conjunctiva.[1] The tear film consists of three interacting layers: an inner mucous layer, a middle aqueous layer, and an outer lipid layer that floats on the middle layer. Different sets of glands are responsible for maintaining each specific layer. The lacrimal gland secretes the proteins, electrolytes, and water that make up the middle aqueous layer.[2,3] The lacrimal gland is made up of acini, ducts, nerves, myoepithelial cells, and plasma cells. The majority, approximately 80%, of the gland are the acinar cells, which secrete electrolytes, water, and proteins.[2,3]

Dry eye syndrome is caused by inadequate quantity or quality of tears. Aqueous-deficient dry eye or keratoconjunctivitis sicca (KCS) is one of two major subtypes of dry eye syndrome, the other being evaporative dry eye.[4–7] Although there are tear substitutes that can be used to temporarily lubricate the eye, there is no curative treatment for dry eye syndrome.[4–6]

Chronic inflammation of the lacrimal gland can lead to insufficient lacrimal gland secretion, leading to symptoms of dry eye.[8–10] Pathologies associated with chronic inflammation, such as autoimmune diseases (Sjögren's syndrome, sarcoidosis, rheumatoid arthritis), organ transplantation, or viral infections (hepatitis, HIV), are often accompanied with symptoms of dry eye syndrome. Lacrimal gland inflammation is characterized by the presence of focal lymphocytic infiltrates, increased production of proinflammatory cytokines, and destruction of the tear-producing parenchymal cells.[8–10]

Development of the lacrimal gland occurs through a process known as branching morphogenesis.[11,12] This process of morphogenesis is achieved through epithelial-mesenchymal interactions that include a highly coordinated spatio-temporal release of several growth factors and subsequent activation of critical transcription factors.[11,12] Several growth and transcription factors have been shown to be crucial for branching morphogenesis of the lacrimal gland. Bone morphogenetic protein (BMP)7 plays a major role in tissue development and remodeling and was shown to play a pivotal role in murine lacrimal gland development.[13] We have recently reported that the BMP7 pathway was not only activated but was upregulated in the lacrimal gland during the repair phase after injury.[14]

The presence of stem cells in adult tissues is a very active area of research because of the potential clinical benefits.[15–18] Several studies have reported the self-regenerating capabilities of salivary glands and the pancreas, liver, intestine, and mammary gland.[19–24] In particular, the regenerative capacity of the salivary glands has been well documented.[22,23,25–30] Long-term (7–21 days) ligation of the main excretory ducts of the salivary glands leads to atrophy of the glands. The ligation of the salivary gland corresponds with inflammation, edema, and death of acinar cells. After the duct ligation is released, the salivary glands go through a phase of increased cellular proliferation and repair. Nestin-positive stem cells have been isolated and propagated in vitro from injured rat pancreas as well as salivary and mammary glands.[22–24,31]

We have recently reported that stem/progenitor cells were present in the murine lacrimal gland and that their number increased during the repair phase after experimentally induced inflammation.[14] These cells were identified by their ability to express nestin, a stem cell marker. Some of the nestin-positive

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cells also expressed α-smooth muscle actin, a marker of myoepithelial cells.[14] This suggested a common source of stem cells between myoepithelial cells and acinar cells in the lacrimal gland; that is, the same stem cell can give rise to both myoepithelial as well as acinar cells. The purpose of the present studies was to determine whether stem/progenitor cells can be isolated from the murine lacrimal gland undergoing repair and if they can be propagated in vitro.

Materials and Methods

Dulbecco's Modified Eagle Medium (DMEM), gentamicin, penicillin-streptomycin, heat-inactivated fetal bovine serum (FBS), trypsin-EDTA, and trypsin replacement (TrypLE Express) solution were obtained from Invitrogen (Carlsbad, CA). Costar culture treated plates were from Corning Life Sciences (Lowell, MA). Tissue culture chamber slides (Laboratory-Tek) were from Nunc, Inc. (Rochester, NY). Collagenase (CLSIII) was purchased from Worthington Biochemicals (Lakewood, NJ). All other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

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), rat monoclonal antibody against ABCG2 (1:20; Abcam, Cambridge, MA), rat monoclonal antibody against Sca-1 (1:20; BD Biosciences, San Jose, CA), and rat monoclonal antibody against Ki67 (1:100; Dako, Carpinteria, CA). Secondary antibodies were from Invitrogen and were conjugated to Alexa Fluor 488 or Alexa Fluor 594.

Animals and Treatment

Female BALB/c mice (10–12 weeks old) were purchased from Taconic (Germantown, NY). Animals were maintained in constant-temperature rooms with fixed light-dark intervals of 12 hours' length and were fed ad libitum. 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–New England Medical Center Animal Care and Use Committee.

Animals were anesthetized, and the exorbital lacrimal glands were left untreated (control) or were injected, in a total volume of 2 μL, with either saline (vehicle) or recombinant human interleukin (IL)-1α (1 μg, a generous gift from Craig W. Reynolds, Biological Resources Branch, National Cancer Institute Preclinical Repository, Rockville, MD), as previously described.[32] Two and half days later, the animals were killed and the lacrimal glands removed. One piece of the lacrimal gland was fixed and processed for histology, immunohistochemistry, or RNA extraction for RT-PCR analysis. The remainder of the tissue was used for explant tissue culture or preparation of acinar cells.

Preparation of Lacrimal Gland Acinar Cells

Lacrimal gland acinar cells were prepared as previously described.[33] Briefly, lacrimal gland pieces (~2 mm) were prepared and incubated in Krebs-Ringer bicarbonate buffer (containing [mM]rsqb]: 119 NaCl, 4.8 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, and 25 $NaHCO_3$) supplemented with 10 mM HEPES, 5.5 mM glucose pH 7.4, 0.5% BSA, and collagenase (150 U/mL). Lobules were subjected to gentle pipetting through tips of decreasing diameter. The preparation was then filtered through nylon mesh (100 μm), and the acini were pelleted by centrifugation (50*g*, 2 minutes).

Tissue Culture

Before culture, lacrimal gland explants and the acinar cells were washed two times in DMEM-containing glucose, 2 mM L-glutamine, and sodium pyruvate and supplemented with 100 μg/mL penicillin/streptomycin, 50 μg/mL gentamicin, and 10% FBS (complete DMEM). Lacrimal gland explants were placed (2 per well) on scored and pre-wet 24-well tissue culture plates (Costar; Corning Life Sciences). The explants were allowed to adhere to the plates for 3 to 4 hours before addition of 0.2 mL of complete DMEM. The medium was replaced every 3 days. The acinar cells were plated (0.2 mL in complete DMEM) into 24-well tissue culture plates (Costar), and the medium replaced every 3 days. Cells were grown under routine culture conditions of 95% air and 5% CO_2 at 37°C. The cells were passaged by trypsinization of confluent, adherent cells with 0.05% trypsin in 0.53 mM EDTA (pH 7.4) or trypsin replacement.

Histopathology and Immunofluorescence

Lacrimal glands pieces were fixed, overnight at 4°C, in 4% formaldehyde in PBS. Paraffin sections of the lacrimal gland (6 μm) were deparaffinized and rehydrated using graded alcohols. For histopathology experiments, paraffin sections of the lacrimal gland were processed for Masson's trichrome staining.

For immunofluorescence experiments, the slides were first subjected to microwave pretreatment (20 minutes) with antigen retrieval solution (Dako). After 3 washes in PBS, nonspecific 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 a mounting medium containing DAPI to stain cell nuclei (Vectashield; Vector Laboratories, Burlingame, CA). Sections were viewed using a microscope equipped for epi-illumination (Nikon UFXII; Nikon, Tokyo, Japan). Omission of the primary antibody or incubation with irrelevant immunoglobulins was performed for negative control experiments.

Adipogenesis

Cells from passage 2 or 3 were trypsinized and seeded into 6-well plates in complete DMEM. When the cells reached 90% confluency, they were either switched to an adipogenic-inducing medium (StemPro Adipogenesis Differentiation Kit; Invitrogen) or kept in complete DMEM. The media was replaced every 2 days. After 21 days of culture, cells were fixed in 4% formaldehyde in PBS (containing in mM: 145 NaCl, 7.3 Na_2HPO_4, and 2.7 NaH_2PO_4 at pH 7.2) for at least 1 hour. Cells were then stained with fresh 0.3% oil Red-O solution.

Table 1. Primer Sequences for the RT-PCR

Gene Name	Forward Primer	Reverse Primer	Product Size (bp)	TM (°C)
Nestin	GGAACCCAGAGACTGTGGAA	CACATCCTCCCACCTCTGTT	167	60
Vimentin	ATGCTTCTCTGGCACGTCTT	AGCCACGCTTTCATACTGCT	206	60
ABCG2	TCGCAGAAGGAGATGTGTTG	TTGGATCTTTCCTTGCTGCT	204	60
Sca-1	ACCTCCACCCTTGTCCTTTT	CTTCACTGTGCTGGCTGTGT	250	60
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452	59

Cell Proliferation

Cell proliferation was determined using a colorimetric assay, cell-counting kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD) for the quantification of cell number and viability. This assay is based on the cleavage, by mitochondrial dehydrogenases in viable cells, of the tetrazolium salt [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (WST-8) to generate a water-soluble formazan dye. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. Lacrimal gland mesenchymal stem cells were trypsinized and seeded on 96-well culture plates at a density of 1000 cells per well. At various time intervals (1, 2, 6, 12, and 14 days), CCK-8 reagent was added, and after 60-minute incubation at 37°C, the absorbance was read at 450 nm in a microplate reader.

RT-PCR

Lacrimal glands were homogenized in reagent (TRIzol; Invitrogen), and total RNA was isolated according to the manufacturer's instructions. RNA was also isolated from mouse brain to use as a positive control and from cultured mesenchymal stem cells derived from IL-1–injected lacrimal glands.

Twenty nanograms of purified total RNA were used for reverse transcription and PCR amplification with a RT-PCR kit (OneStep; Qiagen, Valencia, CA) using primers specific to nestin, vimentin, ABCG2, Sca-1, or G3PDH (Table 1) in a thermal cycler (2720 Thermal Cycler; Applied Biosystems, Carlsbad, CA). G3PDH primers were purchased from IDT (ReadyMade Primers; IDT, Coralville, IA). Primers for nestin, vimentin, ABCG2, and Sca-1 were designed using software (Primer3, http://frodo.wi.mit.edu/primer3/) according to the mispriming library.

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.

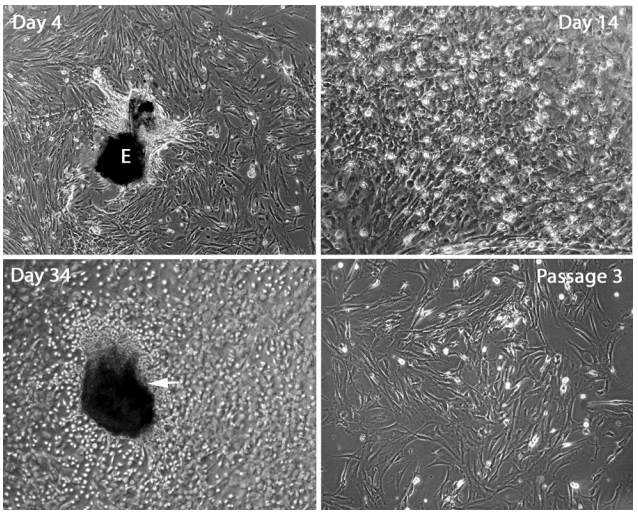

FIGURE 1. Isolation and propagation of stem cells from lacrimal gland explants. Tissue explants were prepared from IL-1–injected lacrimal glands and cultured in complete DMEM. Four days after plating, spindle-shaped, fibroblast-like cells as well as round refringent cells can be seen leaving the tissue explant (E) and adhering to the tissue culture plate. The cells continue to grow and start to form spherical clusters (*arrow*) by day 34, a characteristic of mesenchymal stem cells. These cells can be passaged several times.

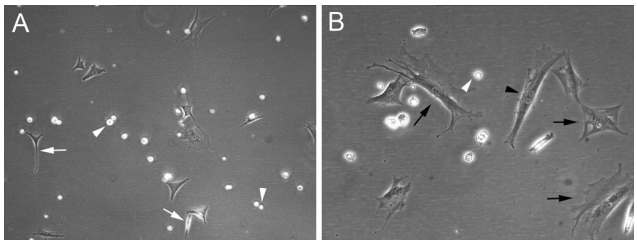

FIGURE 2. Three distinct populations of cells observed in explant cultures. Tissue explants were prepared from IL-1–injected lacrimal glands and cultured in complete DMEM. (**A**) Example of small rounded refringent cells (*arrowheads*) and spindle-shaped cells (*arrows*). (**B**) Example of flattened fibroblast-like cells with a single nucleus (*black arrows*) or binucleate (*white arrows*).

RESULTS

Based on our previous work and on reports in the literature showing that the number of stem cells increases after tissue injury, we used tissue explants prepared from saline (vehicle control) or IL-1–injected lacrimal glands to determine whether stem cells can be isolated and propagated in vitro.

As shown in Figure 1, spindle-shaped and fibroblast-like cells, cuboidal or flattened cells, as well as refringent cells with rounded morphology suggestive of a stem/progenitor cells phenotype can be seen growing out of explants prepared from IL-1–injected lacrimal glands. All these cells appeared as early as the fourth day after plating (Fig. 1). By day 34, the cells formed spherical clusters again a characteristic of cultured mesenchymal stem cells (Fig. 1). Furthermore, the cells continued to grow after multiple passages (Fig. 1). Consistently and especially during early passages, the cultures contained both spindle-shaped cells as well as large flattened cells that were frequently binucleate (Fig. 2). In addition, very small round cells, highly reflective, can be

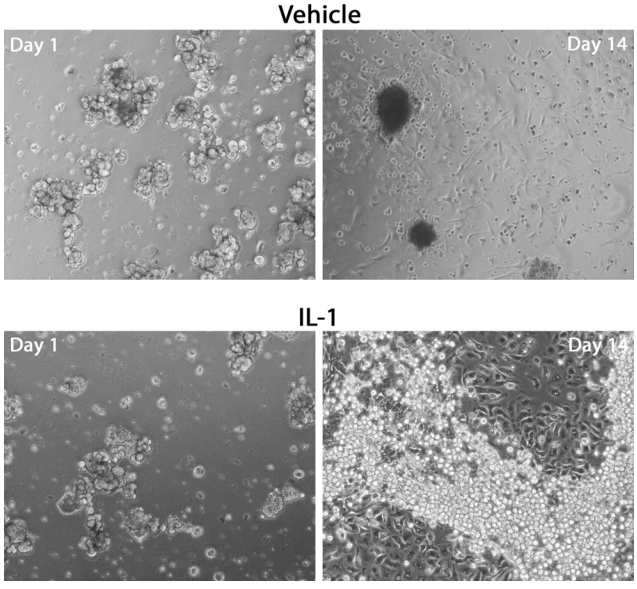

FIGURE 3. Isolation of stem cells from lacrimal gland acinar cell preparations. Partially purified acinar cells were prepared from either vehicle (saline)-injected or IL-1–injected lacrimal glands and cultured in complete DMEM. Although cells prepared from vehicle-injected lacrimal glands adhered to the plate by day 4, they were usually lost by day 14. In contrast, cells isolated from IL-1–injected lacrimal glands adhered to the plate, and after 14 days of culture, spindle-shaped, fibroblast-like, and round refringent cells could be seen.

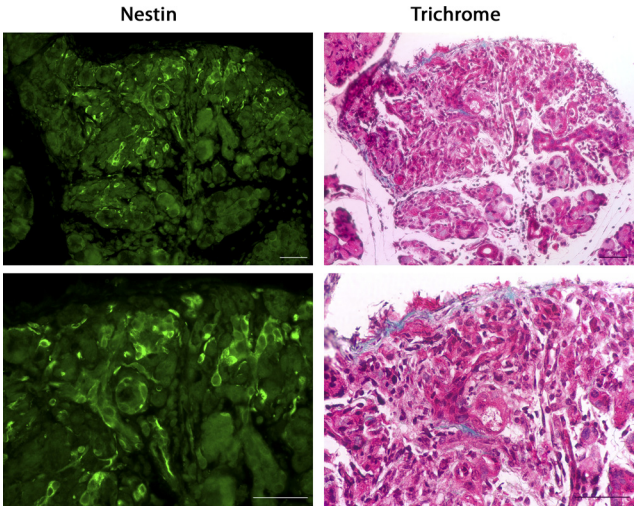

FIGURE 4. Masson's trichrome staining and nestin staining on sequential sections from injured tissue. Lacrimal glands were injured via IL-1 injection and then processed for histopathology and immunohistochemistry. Extracellular matrix formation (*blue*) shown by Masson's trichrome staining corresponds to areas of nestin-positive (*green*) staining. Scale bar, 50 μm.

seen in these cultures (Fig. 2). Cultures of lacrimal gland explants from saline-injected animals showed minimal outgrowth (data not shown).

In another series of experiments, we investigated whether cultures of partially purified acinar cells isolated from IL-1 (and saline)-injected lacrimal glands would yield the same results as tissue explants. As shown in Figure 3, cells prepared from both saline and IL-1–treated lacrimal glands successfully adhered to the culture plate. By the 14th day, adherent cells from IL-1–treated lacrimal glands had heterogeneous shapes with some exhibiting a spindle-shaped morphology whereas others are rounded and highly reflective, similar to the cultures prepared from tissue explants (Fig. 3). These cells could also be successfully passaged (not shown). Although the partially purified acinar cells isolated from saline-injected lacrimal glands tended to adhere to the plate by the fourth day; they were usually lost after the second or third week in culture (Fig. 3). It remains to be determined if the spindle-shaped cells were present along with the partially purified acinar cells during the collagenase digestion or if the acinar cells gave rise to them once they were placed in primary culture. This second possibility seems unlikely since acinar cells from untreated lacrimal glands could not give rise to the spindle-shaped cells.

Taken together, these results show that cells with a mesenchymal stem cell phenotype can be consistently isolated and propagated from injured lacrimal glands undergoing repair using either the tissue explant technique or partially purified acinar cell preparations.

We recently reported that there was an increase in the number of nestin-positive cells in injured glands, indicating initiation of repair of the lacrimal gland.[14] Masson's trichrome staining showed that newly deposited extracellular matrix such as collagen corresponded to areas of nestin-positive staining on lacrimal tissue, attesting to tissue repair and the possible involvement of these cells in the repair process (Fig. 4). We next performed nestin staining on cells isolated from tissue explants and acinar cell cultures derived from injured lacrimal glands. As shown in Figure 5, nearly all cells (passage 3) grown from the explants were nestin positive. Similarly, the majority of the cells derived from the

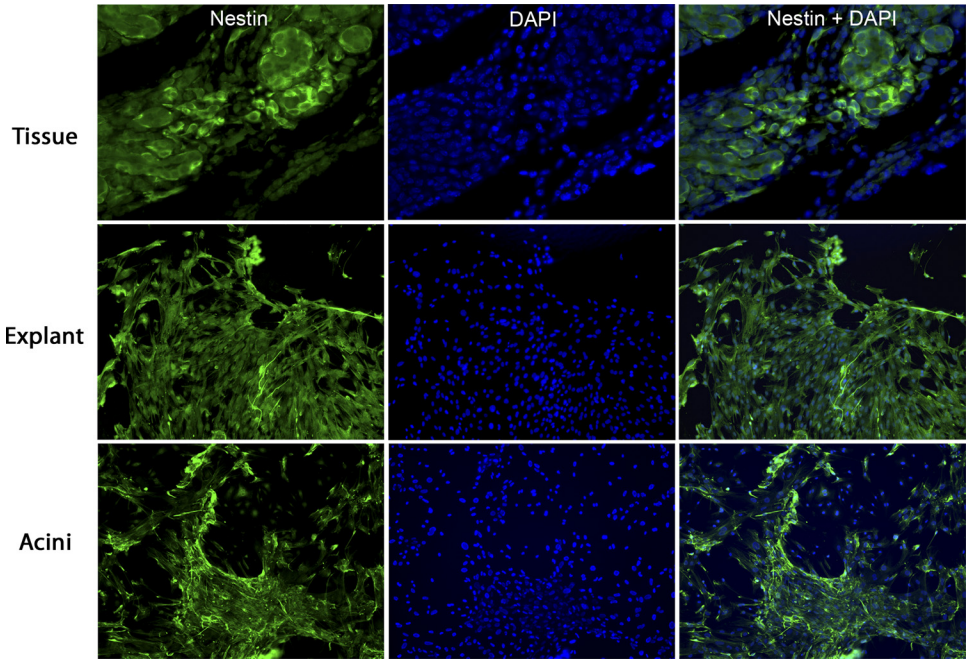

FIGURE 5. Nestin's expression in cultures from explants and partially purified acinar cell preparations. Tissue explants and acinar cells were prepared from IL-1–injected lacrimal glands and cultured in complete DMEM in 8-well chamber slides. Additionally, a piece of the same tissue used for explant or acini preparation was also processed for immunostaining for nestin. Cell nuclei were counterstained with DAPI. Nestin immunoreactivity can be seen in the injured lacrimal gland, attesting to tissue repair. Additionally, the majority of the cells cultured from the explant and from the acinar cell preparation are also nestin positive.

acinar cell preparation were also nestin positive (Fig. 5). For confirmation that the nestin-positive cells were also present in parent tissue, nestin staining was performed on tissue sections from the same gland used for the explants cultures and acinar cell preparation. As shown in Figure 5, nestin-positive cells were indeed present in injured lacrimal glands attesting of initiation of tissue repair.

These results show that nestin immunoreactivity is not lost during isolation and propagation of lacrimal gland stem cells.

We previously reported that during repair of the lacrimal gland, the number of nestin positive cells increased through induction of proliferation.[14] To determine whether cultured nestin-positive cells were proliferating in vitro, we performed double-immunofluorescence staining with antibodies against nestin and Ki67, a protein marker of proliferation that is tightly bound by chromatin in all cells as they progress from the S to the G_2 phase of the cell cycle.[34] As shown in Figure 6A, a fraction of nestin-positive cells were also positive for Ki67, confirming that these cells were proliferating.

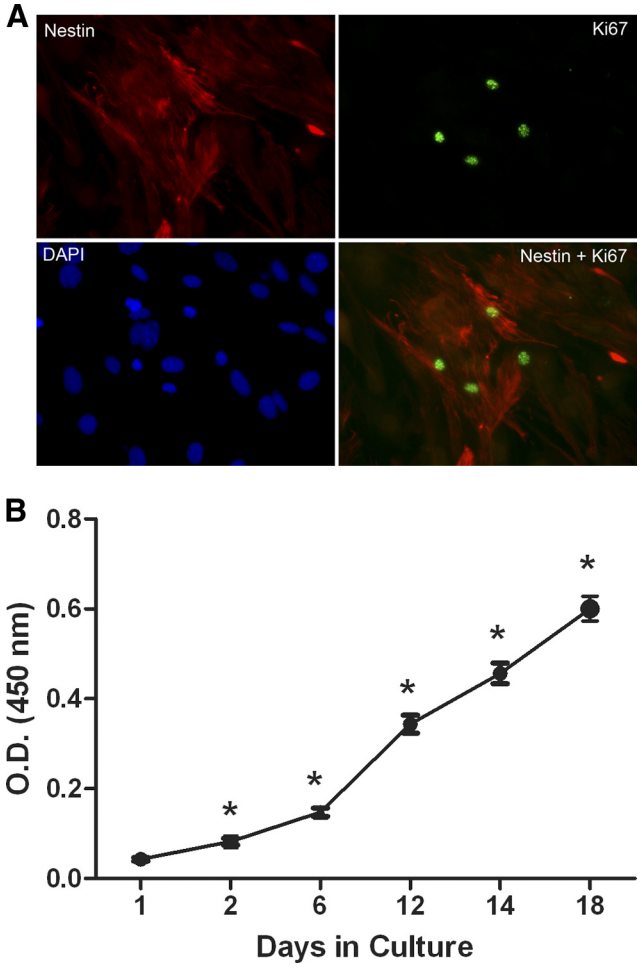

FIGURE 6. Cultured nestin-positive cells proliferate in vitro. (**A**) Passage 3 cells prepared from IL-1–injected lacrimal glands were cultured in 8-well chamber slides in complete DMEM and processed for double immunostaining for nestin and Ki67. Cell nuclei were counterstained with DAPI. Shown are examples of nestin-positive cells that were co-labeled with Ki67. (**B**) Passage 3 cells were trypsinized and seeded on 96-well culture plates at a density of 1000 cells per well. At various time intervals (1, 2, 6, 12, and 14 days in culture), CCK-8 reagent was added, and after 60-minute incubation at 37°C, the absorbance was read at 450 nm in a microplate reader. Data are mean ± SE ($n = 3$). *Statistically significant difference from the 1-day cultures.

As an alternative and to validate the Ki67 findings, we also used a colorimetric assay to quantify cell proliferation. This assay is based on the cleavage, by mitochondrial dehydrogenases in viable cells, of the tetrazolium WST-8, to generate a water-soluble formazan dye. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. As shown in Figure 6B, cells cultured in the presence of 10% FBS continued to grow, and their number continued to increase for up to 18 days.

These results suggest that lacrimal gland mesenchymal stem cells are able to proliferate in vitro.

Mesenchymal stem cells are known to express vimentin, a type III intermediate filament protein.[35–37] To determine whether vimentin was expressed in the lacrimal gland during repair and if its expression persisted in cultured cells, we performed immunohistochemical staining. The same slides were double labeled for nestin expression as a positive control. As shown in Figure 7, nestin expression increased in injured lacrimal glands, attesting to initiation of tissue repair. When the same sections were stained for vimentin, immunoreactivity was intense in areas where nestin staining (i.e., repair) was observed (Fig. 7). When the images were overlaid, it became apparent that some cells did express both types of intermediate filament proteins, whereas others expressed only one of the two (Fig. 7). Similar findings were obtained when cultured cells were probed: Some cells expressed both proteins, whereas others expressed either nestin or vimentin (Fig. 7).

These results suggest that a subpopulation of nestin-positive cells is of mesenchymal origin.

Although the expression of nestin and vimentin suggested that the cells isolated from injured lacrimal glands are stem cells, we investigated the expression of two additional stem cell markers, ABCG2 and Sca-1. ABCG2 (ATP-binding cassette subfamily G member 2), also known as BCRP (breast cancer resistance protein), is responsible for the side population phenotype and is widely expressed in a large variety of stem cells, making it an important stem cell marker. Sca-1 (stem cell antigen 1), a member of the lymphocyte antigen 6 (Ly-6) family, is a glycosyl phosphatidylinositol–linked cell surface protein found on hematopoietic and mesenchymal stem cells. First, we conducted RT-PCR experiments to investigate the gene expression of ABCG2 and Sca-1 using RNA extracted from control lacrimal glands, lacrimal glands injected with IL-1, as well as cultured cells isolated from IL-1 treated glands. As shown in Figure 8A, expression of ABCG2 and Sca-1 mRNAs as well as those for nestin and vimentin was upregulated in IL-1–injected glands compared with control saline-injected ones (lane 3 versus lane 2, Fig. 8A). Furthermore, cells isolated from IL-1–injected lacrimal glands and propagated in vitro (shown are P1, P2, and P3 cells) expressed message for ABCG2 and Sca-1 (Fig. 8A, lanes 4, 5, and 6).

To validate the RT-PCR findings, we performed immunofluorescence staining using rat monoclonal antibodies against ABCG2 and Sca-1. The sections were double-stained for nestin expression, and nuclei were counterstained with DAPI. As shown in Figure 8B, nestin-positive cells were also positive for ABCG2 and Sca-1.

Taken together, these results suggest that the cells isolated from injured lacrimal glands are mesenchymal stem cells.

Another one of the hallmarks of mesenchymal stem cells is their ability to differentiate into osteoblasts and adipocytes. As shown in Figure 9, when lacrimal gland nestin-positive cells were cultured in adipogenesis differentiation media, they dif-

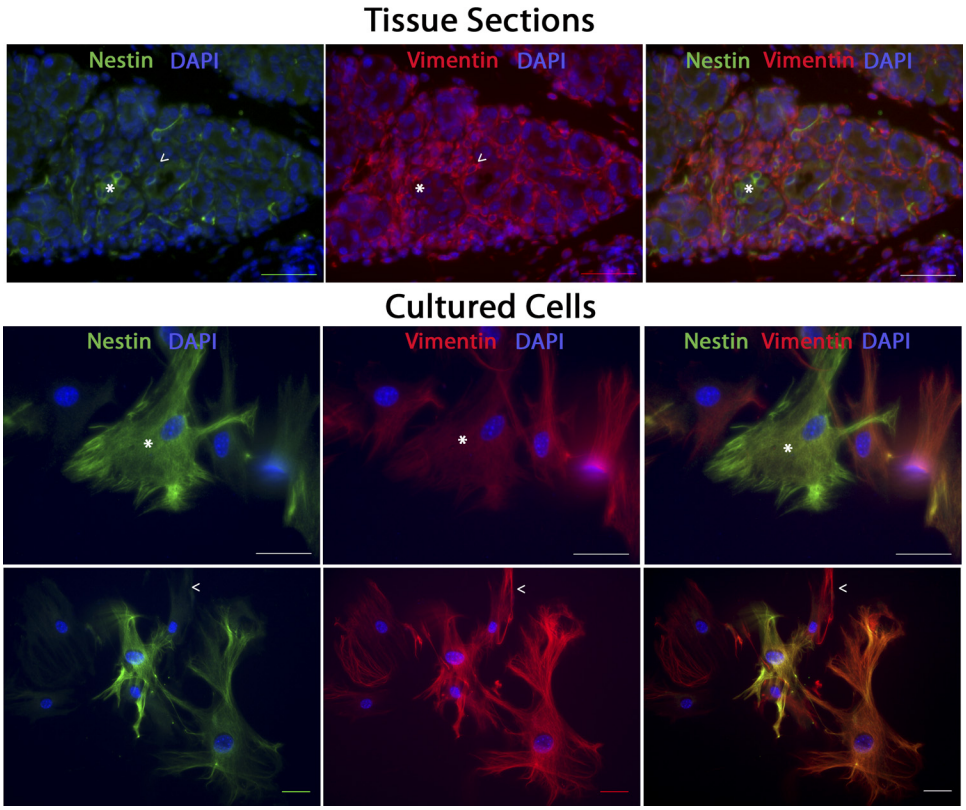

FIGURE 7. Expression of the mesenchymal cell marker vimentin in injured lacrimal glands. IL-1–injected lacrimal glands animals were removed and processed for immunohistochemistry. Passage 3 cells prepared from IL-1–injected lacrimal glands were cultured in 8-well chamber slides in complete DMEM and processed for immunohistochemistry. Both the tissue sections and cultured cells were stained for nestin and vimentin. Cell nuclei were counterstained with DAPI. The staining revealed three populations of cells on the sections and on the cultured cells: nestin-positive cells (*stars*), vimentin-positive cells (*arrows*), and nestin-vimentin double-positive cells. Scale bar, 50 μm.

ferentiated into adipocytes. This result lends further support to the mesenchymal origin of these cells.

DISCUSSION

The presence of stem cells in adult tissue is a very active field of research because of the potential clinical benefits.[15–18] There are two major competing theories in the field of tissue repair: repair by expansion of stem/progenitor cells or repair by transdifferentiation of preexisting cells.[16,21,24,31] One major technical difficulty has been the inability to distinguish between the expansion of stem/progenitor cells and transdifferentiation during tissue repair. Several reports have shown that stem/progenitor cells are involved in repair of salivary and mammary glands and the pancreas, and additionally, these cells could be isolated and expanded in vitro.[19–24] We previously showed that stem cells are also present in the lacrimal gland and could be involved in tissue repair after injury.[14] In studies on the salivary gland, it was reported that the number of stem cells increased after tissue injury, and we reported that this was also the case in the lacrimal gland.[14]

In previous work,[14,32,38] we have developed an animal model of acute, experimentally induced inflammation to study the role of IL-1 in impairing lacrimal gland functions. We showed that a single injection of IL-1 into the lacrimal gland inhibited both neurally as well as agonist-induced protein secretion measured 1 day after injection.[38] We also reported that IL-1 (IL-1β or IL-1α) injection induced a severe inflammatory response in the lacrimal gland that led to destruction of the acinar cells and resulted in reduced aqueous tear production.[32] Furthermore, we showed that the effects of IL-1 on lacrimal gland functions were transient as by the seventh day (BALB/c mice) or 13th day (C57BL/6 mice) after IL-1 injection, inflammation resolved, and the lacrimal gland recovered.[14,32]

In the present study, we present evidence of the ability to consistently isolate and propagate stem cells from injured lacrimal glands. These cells can be isolated using either lacrimal gland tissue explants or purified acinar cell preparations. Spindle-shaped cells and highly reflective rounded cells were consistently obtained in both approaches. These cells can be passaged several times and were capable of congregating and creating spheres characteristic of mesenchymal stem cells. To confirm that these were indeed stem cells, nestin staining was performed and revealed that the majority of cells were nestin positive. Additionally, a subset of these cells also expressed vimentin, another marker of mesenchymal stem cells. Furthermore, these cells expressed two additional markers of stem cells, ABCG2 and Sca-1. Last, these cells could be induced to form adipocytes when cultured in adipogenic media further attesting of their mesenchymal origin.

Induction of tissue repair in the pancreas and the salivary glands relies on the technique of duct ligation. Ligation of the excretory ducts leads to inflammation, edema, and tissue atrophy resulting from death of parenchymal cells.[22–24] After the duct ligation is released, the tissue goes through a phase of increased cellular proliferation and repair that

involves the recruitment of stem/progenitor cells.[22–24] It takes 2 to 3 weeks from the start of duct ligation to induction of tissue repair and recruitment of stem cells.[22–24] In contrast, in our model of experimentally induced lacrimal gland injury, it takes only 2 to 3 days to induce repair and recruitment of stem progenitor cells that can then be easily and consistently isolated in large number using tissue explants. It remains to be tested as to whether or not our mode of tissue injury using injection of IL-1 can be applied to other exocrine glands.

In summary, we present data showing that murine lacrimal glands contain mesenchymal stem cells that can be activated during tissue repair. We also show that these cells can be isolated and propagated in vitro using either lacrimal gland tissue explants or partially purified acinar cells.

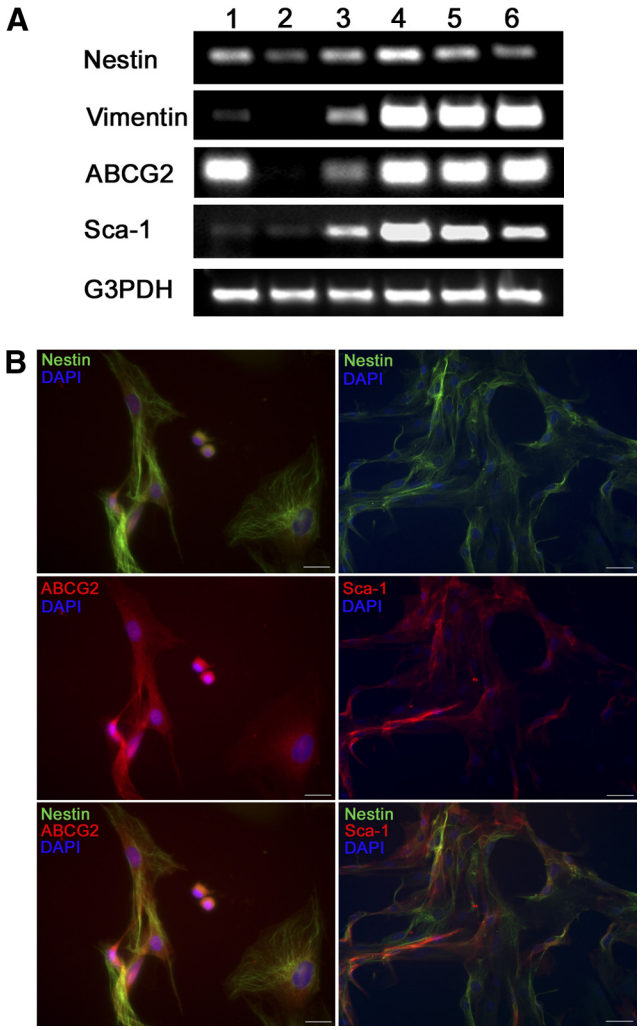

FIGURE 8. Expression of ABCG2 and Sca-1 in injured lacrimal glands and cultured cells. (**A**) RNA isolated from mouse brain (lane 1), control (*lane 2*), or IL-1–injected lacrimal glands (*lane 3*), and P1, P2, and P3 cultured cells (*lanes 4, 5,* and *6*) was used for reverse transcription and PCR amplification using primers specific to nestin, vimentin, ABCG2, Sca-1, or G3PDH (control gene). (**B**) Expression of ABCG2 and Sca-1 in cultured cells. Passage 3 cells prepared from IL-1–injected lacrimal glands were cultured in 8-well chamber slides in complete DMEM and processed for immunohistochemistry. Cells were stained using rat monoclonal antibodies against ABCG2 and Sca-1. Cells were double stained for nestin and cell nuclei were counterstained with DAPI. Scale bar, 50 μm.

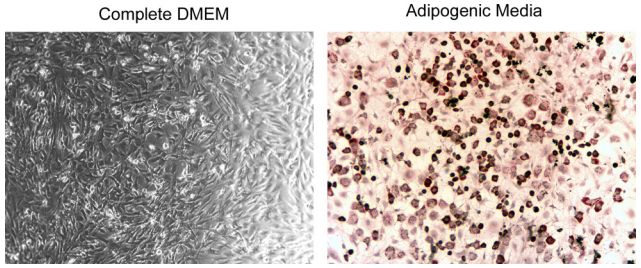

FIGURE 9. Induction of adipogenesis in cells cultured from injured lacrimal glands. Passage 3 cells prepared from IL-1–injected lacrimal glands were cultured in 6-well tissue culture plates in complete DMEM. When the cells reached 90% confluency, they were either kept in complete DMEM or switched to an adipogenic inducing media. After an additional 21 days in culture, cells were fixed and stained with Red-O solution.

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