Keratocytes in the corneal stroma express keratan sulfate-containing proteoglycans including cornea-specific keratan. On plastic dishes, human, bovine, and rabbit keratocytes lose their characteristic dendritic morphology and keratan expression when cultured in serum-containing media. Herein, we demonstrated that keratocytes maintained a normal phenotype while continuously expanded in a serum-containing medium. This strategy of suppressing TGF-β signaling, achieved by AM stromal matrix in part via suppression of TGF-β gene transcription, can be used to expand keratocytes in culture without the use of AM in the future.

Keratocytes, a unique population of neural crest-derived cells embedded in the corneal stroma, play a major role in maintaining corneal transparency. Different culturing methods have been explored to study the mechanism whereby normal keratocytes are regulated in vitro. Bovine (1), rabbit (2), and human (3, 4) keratocytes cultured on plastic rapidly lose their characteristic dendritic morphology and acquire a fibroblastic morphology when exposed to a fetal bovine serum (FBS)-containing medium. Furthermore, these exposed cells down-regulate the expression of keratan sulfate-containing proteoglycans (5–8), keratan (3, 9), and CD34 (4) and up-regulate that of chondroitin-dermatan sulfate-containing proteoglycans (9, 10) and α-SMA (2, 4, 9, 11). Similarly, murine corneal fibroblasts cultured on plastic in a FBS-containing medium lose expression of corneal stroma-specific keratan mRNA but continue to express mimecan and lumican mRNAs (12, 13). To overcome such a detrimental effect of FBS, a serum-free culture system has to be used to maintain a normal dendritic morphology and expression of keratan sulfate-containing proteoglycans at the expense of cell expansion (1).

TGF-β signaling is essential for corneal development and plays a pivotal role in normal and abnormal wound healing (14, 15). One important mediator of TGF-β signaling is the Smad pathway. The binding of TGF-β ligands to TGF-β receptors triggers phosphorylation of Smad 2 and Smad 3, which then translocate into the nucleus to transactivate target genes (16). Therefore, the translocation of these Smads into the nucleus is a common effector in the Smad-mediated pathway (17). In 1% serum or a serum-free medium, addition of TGF-β1 differentiates keratocytes into myofibroblasts with prominent focal adhesions and α-SMA expression and α-SMA, collagen type I and III, fibronectin, paxillin, CD34, and lumican mRNA expression (18). It remains unclear whether suppression of TGF-β signaling is not only important to prevent myofibroblast differentiation but also essential for maintaining the normal keratocyte phenotype.

Recently, we reported a culture system that can achieve effective expansion of human keratocytes by growing them on the stromal surface of the AM in an FBS-containing medium (3, 4). They maintained a dendritic morphology, continued to express corneal stroma-specific keratan for at least 5 passages (at 1:2 split), and did not express α-SMA under serum-containing conditions or on addition of TGF-β1 (4). Previously, we have reported that TGF-β2, TGF-β3, and TGF-βRII is suppressed in both serum-containing and serum-free media, the latter of which was challenged by exogenous TGF-β1, in cultured human corneal and limbal fibroblasts (19) as well as human conjunctival and pterygium fibroblasts (20). Suppression of TGF-β signaling is coupled with down-regulation of α-SMA, in-
Keratocan Maintained by TGF-β Suppression

Transient Transfection and TGF-β Promoter Assays—Freshly isolated cells expanded on AM were subcultured on plastic and AM inserts. Upon reaching 60–80% confluence, cells in each 24-well or AM insert were transfected with 1.0 μg/ml plasmid DNA containing TGF-β2 or TGF-βRII promoter-luciferase and 1.0 μg/ml pCMV/Sport/Bgal (Invitrogen) using GeneJammer® (Stratagene) according to manufacturer’s protocol. The plasmid containing TGF-β2 or TGF-βRII promoter-luciferase was constructed by inserting either human TGF-β2 promoter (~1729 to +63) or TGF-βRII promoter (~1833 to +50) upstream of luc+ in pGL3-basic (Promega, Madison, WI). TGF-β2 promoter (25) was kindly provided by Dr. Seong-Jin Kim (National Institutes of Health, Bethesda, MD) and was inserted into KpnI and HindIII of pGL3-basic. TGF-βRII promoter (26) was amplified by PCR using genomic DNA of human corneal fibroblast as the template, forward primer 5’-GTACGCTAATCCATAGAGTAGTTGTT-3’, and reverse primer 5’-GATCAAGCTTACTCACTTCAACTCAGCCC-3’. The PCR fragment was used 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min. The amplified TGF-βRII promoter fragment was then digested with KpnI and Hind III, gel-purified (Qiagen, Valencia, CA), and inserted at the same sites on pGL3-basic. TGF-β2 and TGF-βRII promoter activities were measured by the Luciferase Assay System® (Promega) and normalized with the β-galactosidase activity.

Adenoviral Transfection—A pKera3.2-intron-ECFP/Bpa plasmid DNA construct was transfected by `In Vitro` Transcription and Translation (Invitrogen) to provide a CMV-EGFP expression cassette. The resulting PCR fragment was digested with EcoRI and Sall simultaneously and then ligated to the EcoRI and Sall sites of the pKera3.2-int-MCS-BPA plasmid vector (12). Fidelity of PCR-amplified EGFP was confirmed by DNA sequencing. Next, the pKera3.2-intron-ECFP/Bpa DNA fragment (6.0 kb) was excised from pKera3.2-intron-ECFP/Bpa plasmid with NotI and KpnI digestion and ligated into pAd-Track plasmid vector, which was kindly provided by Dr. Wei Li (Bascom Palmer Eye Institute, Miami, FL) and contains a CMV-EGFP expression cassette (27). The final construct was designated as pAd-Kera3.2-int-ECFP/Bpa and used to generate recombinant adenoviruses in the 293 cells. When 70% confluent, cells were infected with Escherichia coli according to a previously published method (27) and replication-deficient recombinant adenoviruses in the 293 cells according to previously published method (28). Large scale adenovirus preparation was performed as previously described (12). Purified viruses were aliquoted in 50% glycerol and stored at –80 °C. The viral titer (plaque-forming units/ml) for adenovirus preparation was determined in 293 cell monolayers on a 96-well plate using a standard plaque assay (29).

After 7 days, we checked the green fluorescent protein expression under an inverted fluorescence microscope and estimated titer. The Adenovirus-Track-Kera3.2-int-ECFP/Bpa adenovirus had a titer of 3 × 1011 infectious particles/ml (plaque-forming units/ml). Cells were then transfected by adenovirus-Kera3.2-int-ECFP/Bpa adenovirus (50 pfu per cell) for 24 h. Transfection efficiency was judged by expression of EGFP, and expression of keratocan was judged by expression of ECFP in the same cell using a Nikon-Te-2000u Eclips epifluorescent microscope equipped with appropriate filters.

Immunostaining—To assess protein expression of α-SMA, keratocan, CD34, fibronectin, Smad 2 and Smad 4, culture dishes or frozen sections were fixed in cold methanol for 10 min at –20 °C and blocked and permeabilized as previously described (29). After blocking with 1% bovine serum albumin and 1% goat serum for 30 min, cells were incubated overnight with antibodies to α-SMA (1:100, DAKO), CD34 (1:100), Santa Cruz), fibronectin (1:100, Sigma), Smad 2 (1:50; Santa Cruz), Smad 4 (1:50; Santa Cruz), and keratocan (1:50; rabbit antisemiram against mouse keratocan N-terminal peptide; VRQAYEQPDPEDWD-VHDDFYC; Invitrogen) (27). This peptide was conjugated to a SulfoLink® column (Pierce), which was then used to purify anti-mouse keratocan antibody according to the manufacturer’s instruction. The secondary antibodies (Sigma) used at 1:100 dilution were anti-rabbit, fluorescein isothiocyanate anti-mouse, and fluorescein isothiocyanate anti-goat antibodies for immunofluorescence. ABC method (Vector Laboratories, Burlingame, CA) with 3,3′-diaminobenzidine was applied to visualize primary and secondary staining. Cell nuclei were counterstained with 10 μg/ml Hoechst 33342, propidium iodide, or hematoxylin (all from Sigma).

Reverse Transcription-Polymerase Chain Reaction—Total RNAs were extracted by TRIzol™ reagent from cells cultured on plastic or AM and from two murine corneas as a positive control. Total RNAs equivalent to 1 × 105 cultured cells or 2 corneas were subjected to RT-PCR as recommended by Promega. The final concentration of RT reaction was

Materials and Methods

Isolation and Culture of Keratocytes on Plastic or AM—Six- to 10-week-old CD-1 albino mice were obtained from Charles Rivers (Boston, MA) and handled according to The Association for Research in Vision and Ophthalmology, Inc. (ARVO) guidelines for care of animals in ophthalmic research. After euthanasia, the eyes were encultured by forceps, washed profusely in phosphate-buffered saline, and incubated in DMEM containing 20 μM HEPES, 15 μg/ml dispase II (Roche Applied Science), and 100 μM xanthor at 4 °C for 18 h as previously reported (21, 22). The entire corneal epithelium loosened by this treatment was subsequently removed by vigorous shaking. Under a dissecting microscope, the corneal stroma was separated from the sclera at the corneoscleral limbus by pressing down the limbus with a 27-gauge needle while the eye was held with a forceps. Isolated corneal stromas were incubated overnight at 37 °C in DMEM containing 1.25 mg/ml collagenase (Applied Science) and 0.1 μg/ml gentamicin by HEPS in a non-coated plastic dish until the tissue became “smeared” onto the dish bottom. Digested corneal stromas in collagenase A were centrifuged at 800 × g for 5 min. Keratocytes were resuspended in DMEM containing 20 μM HEPES, ITS (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite), 50 μg/ml gentamicin, and 1.25 μg/ml amphotericin B with or without 10% FBS. This keratocyte-containing cell suspension was then seeded on plastic dishes or on the stromal side of the AM (Bio-Tissue, Miami, FL) fastened to a culture insert as previously described (23).

The suspension of keratocytes prepared from three to four murine corneal buttons was seeded on a 35-mm plastic dish or on the stromal surface of one 32-mm AM insert as previously reported (3). Cells were cultured in DMEM supplemented with 10% FBS (DMEM/10% FBS), and the medium was changed every 2–3 days. When cells reached 80–90% confluence, they were dissociated into single cells by incubation in 0.05% trypsin and 0.53 mM EDTA in Hanks’ balanced salt solution at 37 °C for 5 min in plastic dishes or for 20 min in AM inserts, followed by vigorous pipetting. After centrifuging at 800 × g for 5 min, cells were resuspended in DMEM/10% FBS and seeded on a plastic dish or AM stroma. They were cultured in DMEM containing 10% FBS, 20 μM HEPES, 50 μg/ml gentamicin, and 1.25 μg/ml amphotericin B.

TGF-β1 Challenge and Neutralizing Antibody—To assess whether TGF-β1 affected the cell phenotype, triggered Smad 2 and Smad 4 nuclear translocation, and differentiated keratocytes into myofibroblasts, 10 ng/ml human recombinant TGF-β1 (Sigma) was added to serum-free DMEM/ITS cells for 3 days in DMEM/ITS or DMEM/10% FBS for 24 h, the latter of which was treated with or without 10 μg of a monoclonal antibody neutralizing TGF-β1, TGF-β2, and TGF-β3 (R&D Systems, Minneapolis, MN) per milliliter of DMEM for 48 h before adenoviral transfection.

Morphological Analysis—The corneal stroma after immediate surgical isolation was assessed for cell morphology by phase-contrast microscopy and for viability by Live/Dead Assay® (Molecular Probes, Eugene, OR) according to a previously published method (24) by incubation for 30 min with 2.5 μl of 2 mM calcein-AM and 4 mM ethidium homodimer OR according to a previously published method (24) by incubation for 30 min with 2.5 μl of 2 mM calcein-AM and 4 mM ethidium homodimer in phosphate-buffered saline. Morphology of primary and subcultured cells on plastic and AM was assessed by phase-contrast microscopy. Images were photographed with a Nikon-Te-2000u Eclips epifluorescent microscope (Nikon, Tokyo, Japan).

Assays of Cell Proliferation—To verify that cells indeed proliferated on AM, the passage 2 (P2) cells that were continuously cultured on either AM or plastic were subcultured at a density of 10,000 cells/24-well plastic dish in DMEM/ITS or DMEM/10% FBS or on AM in DMEM/10% FBS. Cells were terminated at day 3 and day 7 for (3,4,5-dimeth-ylthymidine) incorporation (Roche Applied Science) according to the manufacturer’s instruction. This assay (absorbance, 550 nm) yielded a linear correlation for cell numbers above 2500 cells using P2 murine corneal fibroblasts (data not shown). Cells at day 7 were also immunostained using an anti-Ki67 antibody. The number of Ki67-positive nuclei was randomly measured in 10 fields under high magnification (~400) for each culture. Experiments were performed in triplicate.
were found in the cut edge of the excised stroma (Fig. 1C, inset, marked by arrowheads). Keratocytes in the stroma expressed keratocan as evidenced by positive staining with an affinity-purified antibody against mouse keratocan peptide (Fig. 1D). In contrast, corneal epithelial cells or endothelial cells were not stained (Fig. 1D, inset). These results indicated that in vivo murine keratocytes also exhibited a characteristic dendritic morphology and specifically expressed keratocan.

In Vitro Morphology and Proliferation of Keratocytes Cultured on AM—Keratocytes were then isolated from the corneal stroma by collagenase digestion. An average of 5000 cells/mouse cornea was obtained. Cells were seeded at a density of 3000 cells/cm² on either plastic or AM in DMEM/10% FBS. Within 12 h after seeding, cells attached on either substrate and exhibited a distinctly different morphology. Cells on plastic dishes were evenly distributed on the flat surface and adopted a spindle-shaped morphology with a broad stellate cytoplasm (Fig. 2, A and B). They became confluent in 4–5 days. In contrast, cells on AM were dendritic or satellite in shape, had a triangular cell body and a scantly cytoplasm that formed extensive intercellular networks, and projected their dendritic processes in a three-dimensional manner (Fig. 2, D and E). They became confluent in 10 days.

Upon reaching 80–90% confluence, cells expanded on AM or plastic were trypsinized and continuously passaged onto the same type of substrate as used in the primary culture (passage 0, P0). Cells subcultured on plastic at passage 1 (P1) became more flattened (Fig. 2C). In contrast, cells expanded on AM subcultures still maintained a dendritic morphology with pronounced intercellular contacts (Fig. 2F). They continuously preserved such a dendritic morphology until passage 8, when cells became senescent (data not shown). Using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, cells of P2 plastic cultures in DMEM/ITS did not show an increase of cell number during the 1-week period of culturing, whereas cells on plastic in DMEM/10% FBS rapidly expanded in number (Fig. 2G). The number of cells cultured on AM in DMEM/10% FBS was intermediate between the number of cells cultured under the two conditions described above (Fig. 2G, p < 0.05 cf. DMEM/ITS and p < 0.01 cf. DMEM/10% FBS). At day 7, the number of Ki67-positive nuclei in cells cultured on AM in DMEM/10% FBS was significantly greater than that of cells cultured on plastic in DMEM/ITS but less than that of cells cultured on plastic in DMEM/ITS...
cultured on plastic in DMEM/10% FBS (Fig. 2 H, both p < 0.01). Collectively, these results confirmed that cells cultured on AM continued to proliferate and maintained a dendritic morphology in a FBS-containing medium.

**Phenotypic Characterization of Cells Expanded on AM**—To confirm that dendritic cells expanded on AM were indeed keratocytes and not myofibroblasts, immunostaining was performed for the expression of keratocan and α-SMA, respectively. For primary cultures (P0), a majority of dendritic cells cultured on plastic in serum-free DMEM/ITS for 5 days (Fig. 3 A) expressed keratocan (Fig. 3 B) but not α-SMA (Fig. 3 C). In contrast, cells cultured on plastic in DMEM/10% FBS were not dendritic (Fig. 3 D) and did not express keratocan (Fig. 3 E); instead, some cells expressed α-SMA (Fig. 3 F). However, dendritic cells expanded on AM in DMEM/10% FBS (Fig. 3 G) maintained keratocan expression (Fig. 3 H) and did not express α-SMA (Fig. 3 I). In contrast, fibronectin was expressed extracellularly and intracellularly by cells cultured on plastic (Fig. 4 B), but it was not expressed by cells cultured on AM (Fig. 4 D). These results collectively indicate that the keratocyte phenotype was maintained by AM.

To confirm transcript expression of keratocan, total RNAs were extracted from cells on plastic and AM and subjected to RT-PCR. The results showed that the keratocan transcript (size, 1065 bp) was expressed by cells cultured on plastic at passage 0 but was lost at passage 1 (Fig. 5 A) and thereafter (data not shown). In contrast, the keratocan transcript was continuously expressed in an abundant amount from passage 0 to passage 3 (Fig. 5 A) and up to passage 8 (data not shown) when cultured on AM.

To verify the keratocan protein expression, insoluble matrix proteins were extracted by 4 M guanidine HCl and subjected to
Keratocan Maintained by TGF-β Suppression

Expression of keratocan transcript and proteins. A, expression of keratocan transcript (size, 1065 bp) was maintained in AM cultures from P0 to P3. In contrast, such expression was maintained on plastic at P0 but lost at P1. B-actin was used as a loading control. B, cornea stroma extracted with 4 M guanidine HCl from the normal corneal stroma (K), cells cultured on AM at P6 and P8 (designated as A6 and A8 cells, respectively), and cells cultured on plastic at P1 were treated with (lanes D) or without (lanes U) endo-β-galactosidase to remove keratan sulfate and subjected to Western blot using an antibody to murine keratocan. The undigested samples of the normal cornea and A6 and A8 showed high molecular mass smearing, whereas the digested counterparts revealed a 50-kDa band of keratocan (arrow). A similar 50-kDa band was obtained from P2 cultures on AM, but not from P2 cultures on plastic using digestion by keratanase II (Fig. 5B, K II, P2, lanes AM and PL). In contrast, there was no smearing in the undigested sample, nor was the protein band detected after digestion in plastic cultures at passage 1 (Fig. 5B, PL, P1, lanes U and D) and thereafter (data not shown). The negative control of pure AM extract alone without any cultured cells did not contain any keratocan without (data not shown) or with endo-β-galactosidase digestion (Fig. 5B, AM, lane D). Because keratocan expression was strongly observed in the extracellular matrix of in vivo murine corneas, conditioned media from P2 murine keratocyte cultures were also examined for keratocan expression. The result showed that the digested samples of the conditioned medium from AM culture, but not plastic cultures, showed a 50-kDa band.

Western blot analysis using an antibody against the core protein of keratocan. The sample from the normal murine corneal stroma, which was used as the positive control, showed a dense smearing in the high molecular mass region (Fig. 5B, K, lane U). Nevertheless, the same sample after digestion with endo-β-galactosidase showed a positive protein band of ~50 kDa (Fig. 5B, K, lane D). The undigested sample of AM cultures at passages 6 and 8 showed a similar faint smearing in the same high molecular mass region (Fig. 5B, AM, P6 and P8, lanes U, respectively). Both samples after digestion with endo-β-galactosidase showed a strong positive protein band of 50 kDa (Fig. 5B, AM, P6 and P8, lanes D, respectively). A similar 50-kDa band was obtained from P2 cultures on AM, but not from P2 cultures on plastic using digestion by keratanase II (Fig. 5B, K II, P2, lanes AM and PL). In contrast, there was no smearing in the undigested sample, nor was the protein band detected after digestion in plastic cultures at passage 1 (Fig. 5B, PL, P1, lanes U and D) and thereafter (data not shown). The negative control of pure AM extract alone without any cultured cells did not contain any keratocan without (data not shown) or with endo-β-galactosidase digestion (Fig. 5B, AM, lane D). Because keratocan expression was strongly observed in the extracellular matrix of in vivo murine corneas, conditioned media from P2 murine keratocyte cultures were also examined for keratocan expression. The result showed that the digested samples of the conditioned medium from AM culture, but not plastic cultures, showed a 50-kDa band.

Suppression of Smad-mediated TGF-β signaling by AM. A, immunostaining showed that Smad 1 nuclear translocation takes place only in a small percentage of cells (13%) cultured on plastic in DMEM/10% FBS, but this percentage increased to 67% and 85% after 10 ng/ml TGF-β1 was added for 3 h and 5 days, respectively. A similar trend was noted for Smad 2 nuclear localization. B, nuclear localization of Smad 4 was noted in 40% of cells on plastic in DMEM/10% FBS but noted in 10% of cells when TGF-β neutralizing antibody was added for 2 days. C, expression of α-SMA was noted in 39% of cells cultured on plastic after 10 ng/ml TGF-β1 for 5 days, but α-SMA expression was not noted in cells cultured on AM.

To confirm that TGF-β was indeed responsible for Smad signaling in DMEM/10%FBS, we added a neutralizing anti-
body to three TGF-β isoforms in the plastic cultures and noted that nuclear translocation of Smad 4 was indeed prevented (Fig. 6B). To demonstrate whether nuclear localization of Smad 4 also correlated with downstream TGF-β signaling, we quantified α-SMA expression in parallel. Thirty-nine percent of cells cultured on plastic differentiated into α-SMA-expressing myofibroblasts, but no cell on AM expressed α-SMA even after 5 days of continuous stimulation with TGF-β1 (Fig. 6C). Taken together, these results demonstrated that Smad-mediated TGF-β signaling was inhibited in cells cultured on AM and that suppression of Smad-mediated TGF-β signaling correlated with prevention of cells from differentiating into myofibroblasts.

Inhibition of TGF-β2 and TGF-βRII Transcriptional Activity in Keratocytes Cultured on AM—To determine whether the aforementioned down-regulation of TGF-β signaling was mediated by suppressing TGF-β genes at the transcriptional level, TGF-β2 and TGF-βRII promoter activities were evaluated by transient transfection. As compared with cells cultured on plastic and adjusted by background transfection with CMV-β-Gal, the promoter activity of TGF-β2 and TGF-βRII was decreased 4.1-fold and 2.6-fold, respectively, in cells cultured on AM (Fig. 7, both p < 0.001). These data supported the notion that down-regulation of TGF-β signaling was indeed mediated by suppressing TGF-β2 and TGF-βRII genes at the transcriptional level in cells expanded on AM.

Suppression of TGF-β Signaling Maintained Keratocan Expression—To demonstrate a direct link between down-regulation of TGF-β signaling and keratocan expression, 50 multiplicity of infection of Aden-track-Kerapr3.2-intron-ECFP/BpA was added to cells cultured on plastic in either DMEM/ITS or DMEM/10% FBS, the latter of which was further treated with or without an antibody to neutralize all three TGF-β isoforms. Transfection efficiency was revealed by EGFP (green fluorescence) driven by CMV in the same construct, whereas expression of keratocan promoter was revealed by ECFP (blue fluorescence) in the same cell. Cells retained the dendritic morphology after transfection in the positive control cultured on plastic in DMEM/ITS (Fig. 8A) or on AM in DMEM/10% FBS (Fig. 8B). Cells also maintained a flattened bipolar morphology in the negative control cultured on plastic in DMEM/10% FBS (Fig. 8C). These results indicated that transfection itself did not alter their respective characteristic cell morphology. Interestingly, the fibroblastic morphology did not revert to a dendritic morphology after the addition of TGF-β neutralizing antibody for 2 days (Fig. 8D). The overall transfection efficiency was >80% in these experiments (Fig. 8I). Under such a high transfection rate, keratocan promoter-driven ECFP expression was observed in 30–40% of cells cultured on plastic in DMEM/ITS (Fig. 8E) and 15–20% of cells cultured on AM in DMEM/10% FBS (Fig. 8F) but in <2% of cells cultured on plastic in DMEM/10% FBS (Fig. 8G). These results corroborated with the aforementioned pattern of keratocan transcript and protein expression in these three cultures (Fig. 5). ECFP expression was restored to 10–15% of cells cultured on plastic in DMEM/10% FBS when TGF-β neutralizing antibody was added (Fig. 8H). Collectively, these results indicated that TGF-β in 10% FBS was indeed responsible for the suppression of keratocan expression for cells cultured on plastic and that keratocan expression by cells cultured on AM in 10% FBS was correlated with suppression of Smad-mediated TGF-β signaling.

**DISCUSSION**

In this report, we have demonstrated that murine keratocytes expanded in an FBS-containing medium preserved a native three-dimensional dendritic morphology with extensive intercellular contacts and continuously expressed keratocan and CD34, but not fibronectin and α-SMA, for up to 8 passages,
as long as they were cultured on AM stromal matrix (Figs. 2–4). These findings are in agreement with what we have reported in human corneal keratocytes (3, 4). Previously, it has been noted that other small leucine-rich proteoglycans are maintained or up-regulated in plastic cultures of murine fibroblasts at late passages (12, 13). We noted herein that expression of keratocan transcript and protein was lost by cells cultured on plastic in the presence of 10% FBS as early as passage 1 but was continuously maintained by cells on AM for up to passage 8 (at 1:2 split) before senescence (Fig. 5). Furthermore, based on the digestibility with endo-β-galactosidase and keratanase II, keratocan expressed by these keratocytes expanded on AM was glycosylated with keratan sulfate, secreted into the conditioned medium, and deposited in the extracellular matrix (Fig. 5). This feature is known to be unique to keratocytes in the corneal stroma (31). In the presence of 10% FBS, cells cultured on AM were indeed proliferating to a lesser extent than their counterparts cultured on plastic (Fig. 2). Therefore, we conclude that the phenotype of murine keratocytes, defined by a dendritic morphology, positive expression of keratan sulfate-containing keratocan and CD34, and negative expression of fibronectin and α-SMA, is maintained on AM even when cells are stimulated to proliferate by 10% FBS.

We speculate that TGF-β is a factor in FBS that is responsible for the loss of the normal keratocyte phenotype when cells are cultured on plastic dishes. TGF-β is well known for its action in stimulating fibroblasts to differentiate into myofibroblasts (32, 33). Nevertheless, it remains unknown whether suppression of TGF-β signaling, which can prevent myofibroblast differentiation, is also essential for maintaining the normal keratocyte phenotype. Although an earlier study showed that addition of TGF-β1 in DMEM supplemented with 1% platelet-poor horse serum (deficient in TGF-β1) decreases the expression of keratan sulfate proteoglycans at the transcript and protein levels in bovine cells (9, 10), expression of keratocan was not defined in these two studies. In the present study, we noted that the keratocyte phenotype correlated not only with negative expression of α-SMA and fibronectin expression, i.e. myofibroblast differentiation (Figs. 3 and 4), but also with suppression of Smad-mediated TGF-β signaling (Fig. 6). It is known that Smad-mediated TGF-β signaling, judged by nuclear translocation of Smad 2 and Smad 4, takes place in less than an hour and is maximized in 3–4 h (34). However, subsequent interplay and cross-talk between Smad-mediated signaling and other signaling pathways can modify the final outcome of transactivation of targeted genes (for review, see Refs. 32 and 33). That was why we examined nuclear localization of Smad 2 and Smad 4 not only at 3 h but also at 5 days to make sure that cross-talks and interplays with other signaling pathways would not deter Smad signaling. Our results showed that following exogenous addition of 10 ng/ml TGF-β1 in DMEM/ITS, the proportion of cells exhibiting nuclear translocation of Smad 2 and Smad 4 on plastic cultures continuously increased from 3 h to 5 days presumably due to TGF-β1 autoinduction (35), eventually resulting in expression of α-SMA in 5 days (Fig. 6). Chronic nuclear localization of Smads ensures expression of Smad-targeted genes including α-SMA (36, 37). Indeed, such chronic nuclear location of Smad 2 and Smad 4 correlated with eventual expression of α-SMA gene in 5 days (Fig. 6) and up-regulation of TGF-β2 and TGF-βRII promoter activities (Fig. 7). On the contrary, if TGF-β signaling is interrupted or modified by other signaling pathways, the nuclear translocation of Smad will be transient (for review, see Refs. 32 and 33).

In this study, however, we showed that nuclear exclusion of Smad 2 and Smad 4 occurred at both 3 h and 5 days in cells cultured on AM, eventually leading to suppression of α-SMA (Fig. 6). Furthermore, by suppressing Smad-mediated signaling to the nucleus, subsequent transcription of TGF-β2 and TGF-βRII was also aborted, as evidenced by their promoter activities (Fig. 7). These results strongly support the notion that suppression of Smad-mediated TGF-β signaling by AM is potent, complete, and sustained for a prolonged period of time despite exogenous challenge of TGF-β1. They also explain why clinical transplantation of AM is effective in reducing scar formation during ocular surface reconstruction in ophthalmology (for reviews, see Refs. 38–40).

To draw a causative link between suppression of Smad-mediated TGF-β signaling and keratocan expression, we added TGF-β neutralizing antibody to the plastic cultures in DMEM containing 10% FBS. Such a maneuver effectively blocked Smad 4 nuclear translocation (Fig. 6B) and, at the same time, up-regulated keratocan promoter activity (Fig. 8). Collectively, these findings further support that suppression of Smad-mediated TGF-β signaling is pivotal not only in preventing myofibroblast differentiation, but also in sustaining the keratocan-expressing keratocyte phenotype. From such a strategy of suppressing TGF-β signaling, we have developed a serum-free medium to promote keratocyte proliferation while maintaining their normal phenotype.2 Furthermore, our preliminary studies showed that such suppression of TGF-β signaling was retained in soluble AM extracts (data not shown), suggesting that this action does not require cell-matrix contact. Recently, we reported that soluble non-glycosylated lumican isolated from AM promotes mouse corneal wound healing (41). Investigation is under way to identify the exact molecular component(s) in AM soluble extracts that is responsible for down-regulating TGF-β signaling so that we might develop new therapeutics in combating scar formation in the future.

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*J. Biol. Chem.* 2005, 280:27085-27092.
doi: 10.1074/jbc.M409567200 originally published online May 20, 2005

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