Published in final edited form as:

Invest Ophthalmol Vis Sci. 2006 May ; 47(5): 1918–1927.

Preservation and Expansion of the Primate Keratocyte Phenotype by Downregulating TGF- β Signaling in a Low-Calcium, Serum-Free Medium

Tetsuya Kawakita¹, Edgar M. Espana¹, Hua He¹, Robert Smiddy¹, Jean-Marie Parel², Chia-Yang Liu², and Scheffer C. G. Tseng¹

¹ From TissueTech, Inc., Ocular Surface Center, Miami, Florida; and the

² Ophthalmic Biophysics Center, Bascom Palmer Eye Institute, University of Miami School of Medicine, Miami, Florida.

Abstract

Purpose—To demonstrate whether the original keratocyte phenotype is maintained with proliferative activity by suppressing TGF- β signaling in rhesus monkey keratocytes expanded in a serum-free and low-[Ca²⁺] medium.

Methods—Rhesus monkey keratocytes were isolated from central corneal buttons by collagenase digestion for 16 hours, seeded on plastic in Dulbecco's modified Eagle's medium (DMEM) containing insulin-transferrin-sodium selenite (ITS) supplement (DMEM/ITS) or 10% fetal bovine serum (DMEM/10% FBS), or in a defined keratinocyte serum-free medium (KSFM). After confluence, cells in KSFM were continuously subcultured at a 1-to-3 split. Cellular proliferation was analyzed by immunostaining for Ki67 and the MTT assay. The cellular phenotype was determined by immunostaining for aldehyde dehydrogenase (ALDH), keratocan, and CD34 and by the expression of keratocan promoter-driven enhanced cyan fluorescent protein (ECFP). The stability of the keratocyte phenotype was examined by switching KSFM to DMEM/ITS and DMEM/10% FBS. TGF- β signaling was monitored by measuring the promoter activity of TGF- β 1, - β 2, and - β RII after transient adenoviral transfection, and cytolocalization of Smad2 and Smad4.

Results—In KSFM, monkey keratocytes proliferated while maintaining the expression of keratocan, CD34, and ALDH proteins and keratocan promoter-driven ECFP for at least 15 passages. The nuclear accumulation of Smad2 and Smad4 and the promoter activities of TGF- β 1 and - β RII were significantly down-regulated in KSFM compared with DMEM/10% FBS. In KSFM, an increase of [Ca²⁺] to 1.8 mM and addition of 10% FBS synergistically downregulated the keratocan promoter activity, facilitated Smad2 and Smad4 nuclear translocation, and upregulated TGF- β 1 and - β RII promoter activities.

Conclusions—The normal monkey keratocyte phenotype can be maintained in a low-calcium, serum-free medium by downregulating Smad-mediated TGF- β signaling.

Keratocytes, a unique population of neural crest-derived cells embedded in the corneal stroma, play a major role in maintaining corneal transparency. They are mitotically quiescent, exhibit

Corresponding author: Scheffer C. G. Tseng, Ocular Surface Center, 7000 SW 97 Avenue, Suite 213, Miami, FL 33176; stseng@ocularsurface.com..

Disclosure: **T. Kawakita**, TissueTech, Inc. (E, F); **E.M. Espana**, TissueTech, Inc. (E, F); **H. He**, TissueTech, Inc. (E, F); **R. Smiddy**, TissueTech, Inc. (E, F); **J.-M. Parel**, TissueTech, Inc. (F); **C.-Y. Liu**, TissueTech, Inc. (F); **S.C.G. Tseng**, TissueTech, Inc. (E, F)

Supported by TissueTech, Inc., Ocular Surface Research and Education Foundation, Miami, Florida; National Eye Institute Grants EY06819 and EY15735 (SCGT) and EY12486 (C-YL); the Florida Lions Eye Bank (J-MP); and the Henri and Flore Lesieur Foundation (J-MP).

a dendritic morphology with extensive intercellular contacts,^{1,2} and express keratan sulfate-containing proteoglycans (KSPGs),^{3,4} aldehyde dehydrogenase (ALDH)⁴ and CD34.^{5–7} When a scar forms during corneal wound healing, keratocytes turn into fibroblasts by losing the aforementioned morphology and downregulating the expression of KSPG^{8,9} and CD34,⁵ and eventually they differentiate into myofibroblasts^{10,11} that express α -smooth muscle actin (α -SMA), fibronectin, and biglycan.⁴

The aforementioned abnormal phenotypic changes can be observed in vitro on plastic dishes by adding fetal bovine serum (FBS). For example, the characteristic dendritic morphology of bovine,¹² rabbit,^{13,14} and human^{7,15} keratocytes is maintained in a serum-free medium. Nevertheless, when FBS is added, cells take on a flattened fibroblastic morphology and lose the expression of KSPG, including corneal stroma-specific keratocan.^{4,7,15–19} Unfortunately, keratocytes cultured in the serum-free medium do not proliferate.

Therefore, expanding keratocytes while maintaining their normal phenotype in vitro is very desirable. Because FBS contains TGF- β ,²⁰ which promotes myofibroblast differentiation,^{4,14,21,22} many have speculated that TGF- β signaling is responsible for promoting myofibroblast differentiation from keratocytes. It remains unknown whether suppression of TGF- β signaling is not only sufficient to prevent myofibroblast differentiation but also essential for maintaining the keratocyte phenotype. Because expression of the TGF- β 2, - β 3, and - β RII transcripts is suppressed when human corneal and limbal fibroblasts²³ and human conjunctival and pterygium fibroblasts²⁴ were cultured on human amniotic membrane (AM) stroma, we used AM as a culturing substrate to maintain a dendritic morphology and expression of keratocan and CD34 by human keratocytes while continuously stimulating them to proliferate in a medium containing 10% FBS.^{7,15} Extracellular calcium concentration ($[Ca^{2+}]$) significantly affects TGF- β signaling. For example, increased $[Ca^{2+}]$ promotes expression of TGF- β in human vascular endothelial cells.²⁵ Chelation of extracellular $[Ca^{2+}]$ blocks TGF- β -mediated $[Ca^{2+}]$ influx and calcineurin (calcium-dependent signaling intermediate) activity,²⁶ and inhibits TGF- β -mediated α -SMA promoter signaling in human embryonic lung fibroblasts.²⁷ With this $[Ca^{2+}]$ effect in mind, we examined in the current study whether the phenotype of keratocytes can also be maintained on plastic by suppressing TGF- β signaling with a low- $[Ca^{2+}]$, serum-free medium supplemented with growth factors.

Materials and Methods

Reagents

The tissue culture plastic plates (96-well and 6-well) were purchased from BD Biosciences (Lincoln Park, NJ); collagenase A and dispase II powder from Roche (Indianapolis, IN); amphotericin B, Dulbecco's modified Eagle's medium (DMEM), defined keratinocyte serum-free-medium (KSFM), FBS, gentamicin, Hanks ' balanced salt solution (HBSS), HEPES buffer, phosphate-buffered saline (PBS), soybean trypsin inhibitor, and 0.25% trypsin/1 mM EDTA were purchased from Invitrogen-Gibco (Grand Island, NY); A cell-viability–cytotoxicity kit (Live/Dead) from Invitrogen (Eugene, OR); endo- β -galactosidase was from Seikagaku (Tokyo, Japan); optimal cutting temperature (OCT) from Sakura Finetek (Torrance, CA); and a cell-proliferation assay (MTT) from Roche. Other reagents and chemicals including bovine serum albumin (BSA) and insulin-transferrin-sodium selenite (ITS) medium supplement were from Sigma-Aldrich. The monoclonal antibodies against CD34 (QEnd 10) and Ki67 (MIB-1) were from Dako (Carpinteria, CA), and that against ALDH (clone 44) was from BD Biosciences (San Jose, CA). The polyclonal antibodies against Smad2 (S-20) and Smad4 (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of an Epitope-Specific, Polyclonal, Anti-human Keratocan Antibody

Rabbit anti-human keratocan (hKera) peptide antiserum was obtained from EvoQuest Custom Antibody Services (Invitrogen Corp., Carlsbad, CA). In brief, an oligopeptide with the sequence of CPSPSMLPAERDS-FSYGPHL, corresponding to the C-terminal amino acid residues deduced from human lumican cDNA (GenBank NM_007035; <http://www.ncbi.nlm.nih.gov/GenBank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) was synthesized and coupled to a maleimide-activated carrier protein, keyhole limpet hemocyanin (KLH). The KLH-conjugated peptide was then used to raise antiserum in rabbit. To purify the anti-hKera antibody, the CPSPSMLPAERDSFSYGPHL oligopeptide was immobilized by covalent reaction with iodoacetyl groups on gel (Sulfolink; Pierce, Rockford, IL). The rabbit antiserum was loaded onto a peptide-conjugated gel column, according to the manufacturer's instructions. Fractions containing purified anti-lumican antibody were pooled and concentrated. The protein concentration was measured by spectrophotometry at OD_{280nm}. The specificity of the anti-hKera antibody was evaluated by Western blot analysis.

Isolation and Culture of Rhesus Monkey Keratocytes

Animals used in this study were handled according to the guidelines described in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Three rhesus monkeys (*Macaca mulatta*), 4 years old, and rabbit and mouse corneas were obtained from an approved tissue-sharing program after euthanasia (University of Miami Institutional Animal Care and Use Committee approval 04-073). An entire anterior corneoscleral segment was removed from the globe by cutting near the limbus with Wescott scissors. A central cornea was obtained with an 8.0-mm Barron trephine and immediately transferred to KSFM (cat. no. 17005-042; Invitrogen-Gibco). After removing Descemet's membrane and the corneal endothelium, the corneal epithelium was removed by dispase digestion for 16 hours at 4°C, and the remaining corneal stroma was incubated at 37°C for 16 hours in 2.5 mL of DMEM containing 1 mg/mL collagenase A, 20 mM HEPES, 50 µg/mL gentamicin, and 1.25 µg/mL amphotericin B in a plastic dish. Afterward, cells were resuspended in 1 mL of KSFM, centrifuged to remove residual matrices, resuspended in KSFM, and seeded on plastic dishes in KSFM or DMEM containing insulin, transferrin, and selenium supplement (DMEM/ITS; cat. no. 41400-045; Invitrogen-Gibco) or 10% FBS (DMEM/10% FBS).

When the primary culture on plastic reached 80% confluence, cells were rendered into single cells by incubation in BSS containing 0.25% trypsin/1 mM EDTA at 37°C for 1 to 5 minutes, and the enzymatic reaction was stopped by adding soybean-trypsin inhibitor. After they were centrifuged at 800g for 5 minutes, the cells were resuspended in KSFM, subdivided into three equal parts, and seeded on plastic dishes. They were cultured in KSFM continuously until use. Keratocytes were similarly isolated from mouse, rabbit, and human corneas and cultured in KSFM for comparison.

Cell Proliferation

Cell proliferation in KSFM was verified by subculturing primary cells in DMEM/ITS, DMEM/10% FBS, or KSFM at a density of 3000 cells per 96-well plastic dish, and subjected at days 3 and 7 to the MTT assay (Promega Corp., Madison, WI), according to the manufacturer's instructions. Briefly, this assay measures mitochondrial dehydrogenase enzyme activity using the substrate of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at absorbance of 50 nm, to reflect the proliferative activity of viable cells. Using the culture medium alone as the negative control, we had validated this assay by establishing a linear correlation between 2,500 and 10,000 passage-2 murine corneal fibroblasts (data not shown). Cells at day 7 were also immunostained with an anti-Ki67 antibody (1:100). The number of Ki67-positive nuclei was randomly measured in 10 fields under high magnification (400×) for each culture and the

ratio of positive cells to total cells calculated in each field. Experiments were performed in triplicate.

Morphologic Analysis and Immunostaining

The cell morphology was documented by phase-contrast microscopy, and cell–cell contacts were analyzed by staining with a cell-viability assay (Live/Dead; Invitrogen), with methods similar to those in a prior report.² The latter staining enhanced the visibility of the keratocyte network and morphology in our recent studies of human keratocytes.¹⁵

The cell phenotype was studied by immunostaining. These cultures and frozen sections of the cornea (4 μ m thickness) were fixed with cold methanol (-20°C) for 5 minutes. After they were blocked with 1% BSA for 30 minutes, the cells were incubated for 1 hour with antibody against CD34 (1:40), keratocan (1 $\mu\text{g}/\text{mL}$), ALDH (1:100), Smad2 (1:100), and Smad4 (1:100).

Immunoreactivity was detected by ABC kit (Vectastain Elite; Vector Laboratories, Burlingame, CA) using a DAB kit (Dako), or by immunofluorescence staining using appropriate FITC-conjugated secondary antibody, and photographed with an epifluorescence microscope (Te-2000u Eclipse; Nikon, Tokyo, Japan). For Smad staining, clear nuclear accumulation was counted in 10 random fields under high magnification (400 $\times$).

Western Blot Analysis

Monkey corneas or cultured keratocytes were homogenized in extraction buffer containing 4 M guanidine-HCl containing 10 mM sodium acetate, 10 mM sodium EDTA, 5 mM aminobenzamidine, and 0.1 M ϵ -amino-*n*-caproic acid. The extracts were dialyzed exhaustively overnight against distilled water, and the water-insoluble fraction was dissolved in 0.1 M Tris acetate solution (pH 6.0) containing 6 M urea. The protein concentration was measured by spectrophotometer at OD_{280nm}. Protein aliquots (100 μg) were incubated with 0.1 U/mL endo- β -galactosidase (Seikagaku) or 0.1 U/mL keratanase II (Seikagaku) in PBS at 37°C overnight, to remove the keratan sulfate (KS) chain. Each sample was added with an equal volume of 2 $\times$ SDS sample buffer, boiled for 5 minutes, electrophoresed on a 4% to 15% gradient SDS-PAGE gel, and transferred to a nitrocellulose membrane. These membranes were preincubated with the blocking buffer, probed with affinity-purified antiserum against human keratocan and alkaline phosphatase-conjugated goat anti-rabbit IgG (Pierce) as a secondary antibody, and visualized (Western Blue; Promega).

Adenoviral Transfection

Freshly isolated cells expanded in KSFM were subcultured on plastic, and, when 60% to 80% confluent at passage 1, were placed in 96-well plates in KSFM and cultured for 7 days. In parallel cultures, $[\text{Ca}^{2+}]$ was increased to 1.8 mM in KSFM by adding CaCl_2 (identical with that in DMEM) with or without 10% FBS, culture medium was changed to DMEM/10% FBS. Cells were transfected (200 multiplicities of infection [MOI]) for 24 hours with aden-track-Kerapr3.2-intron-ECFP/BpA adenovirus, which was constructed by insertion of an enhanced cyan fluorescent protein (ECFP) fragment generated by PCR using pECFP-N1 (BD-CloneTech, Palo Alto, CA) as the template and two restriction enzyme site-tagged primers (ECFP-RI, 5'-GATCGAATTCCCACCGGTCGCCACCATGGTG-3' and ECFP-SalI, 5'-GTTACTCGACTTACTTGTACATCTCGTCCATG-3'). The resultant PCR fragment was digested and then ligated to the *Eco*RI and *Sal*I sites of the pKera3.2-int-MCS-BPA plasmid vector.²⁸ The fidelity of PCR-amplified ECFP was confirmed by DNA sequencing. The Kerapr3.2-intron-ECFP/BpA DNA fragment (6.0 kb) was excised from the pKerapr3.2-intron-ECFP/BpA plasmid with *Not*I and *Kpn*I digestion and ligated into pAd-Track plasmid vector, which was kindly provided by Wei Li (Bascom Palmer Eye Institute, Miami, FL) and contains a CMV-EGFP expression cassette.²⁹ The final construct was designated as pAd-Kerapr3.2-intron-ECFP/BpA and used to generate a replication-defective adenoviral plasmid by

homologous recombination in *Escherichia coli* according to a previously published method.^{28–30} Purified viruses were aliquotted in 50% glycerol and stored at -80°C . The viral titer (plaque forming units [PFU] per milliliter) for adenovirus preparation was determined in strain 293 cells on 96-well plates with a serially diluted virus used for transfection. After 7 days, we checked the GFP expression under an inverted fluorescence microscope and obtained the estimated titer. The Aden-track-Kerap3.2-intron-ECFP/BpA adenovirus had a titer of 3×10^{11} infectious particles per milliliter (PFU/milliliter). Transfection efficiency was judged by expression of EGFP and expression of keratocan by expression of ECFP in the same cell, by an epifluorescence microscope (Te-2000u Eclipse; Nikon) equipped with the appropriate filters. Ten different fields (400 $\times$) were randomly selected for counting the number of cells with EGFP or ECFP expression.³¹

Passage-1 cells were also transfected with replication-defective adenoviruses containing TGF- β 1 or TGF- β RII promoters, each linked with luciferase (100 MOI) and containing CMV- β -galactosidase (30 MOI) for 48 hours. The medium was replaced with fresh KSFM, DMEM/ITS, or DMEM/10% FBS 5 hours before transfection. The plasmid containing TGF- β 1 promoter-luciferase was provided by Seong-Jin Kim (National Institutes of Health, Bethesda, MD). TGF- β RII promoter³² was amplified by PCR with genomic DNA of human corneal fibroblast used as the template, the forward primer of 5'-GTACGGTACCCATCAAAGAAGTTATGGTTC-3' and the reverse primer of 5'-GTACAAGCTTACTCAACTTCAACTCAGCGC-3' using the PCR program of 95°C , 30 seconds; 55°C , 30 seconds; and 72°C , 2 minutes for 30 cycles. The amplified TGF- β RII promoter fragment was then digested with *Kpn*I and *Hind*III, gel purified (Qiagen, Valencia, CA), and inserted into the same sites of pGL3-basic. The plasmid containing TGF- β RII promoter-luciferase was constructed by inserting TGF- β RII promoter (−1883 to +50) upstream of *Luc*⁺ in pGL3-basic (Promega). Replication-defective adenoviruses were generated for each promoter contrast by the Core Laboratory of the University of Michigan, according to a previously published method.^{29,30} The promoter activity was measured (Luciferase Assay System; Promega) and normalized to β -galactosidase activity. In same manner, the TGF- β 1 and - β RII promoter activity of passage-1 cells was measured in cells cultured in KSFM, in which the $[\text{Ca}^{2+}]$ was increased to 1.8 mM (identical with DMEM), with or without 10% FBS. β -Galactosidase activity was measured, and relative transfection was normalized by using a method previously described.³¹

Statistical Analysis

The significance of differences between groups was determined by either Student's *t*-test. Repeated-measures ANOVA was used when more than one comparison was made. $P < 0.05$ was considered statistically significant.

Results

Phenotype of Rhesus Monkey Keratocytes In Vivo

The cellular morphology of rhesus monkey keratocytes in vivo was studied by phase-contrast microscopy and a cell viability assay, with the latter demarcating the entire cytoplasm. Similar to what has been reported in human keratocytes,¹⁵ monkey keratocytes showed a compact cell body with long dendritic cytoplasmic processes connecting with neighboring cells (Figs. 1A, 1B). These processes formed extensive intercellular contacts in a three-dimensional pattern. In addition, CD34 was clearly expressed in the cytoplasm of these cells using both immunohistochemistry and immunofluorescence staining (Fig. 1C). Western blot showed that the affinity-purified polyclonal antibody against human keratocan also cross-reacted with monkey keratocan (Fig. 1D). This cross-reactivity was attributed to the fact that there is a 92.5% to 95% homology between human and rhesus monkey keratocan genes.³³ Western analysis

showed a blot in the high-molecular-weight region in undigested samples (u) consistent with the nature of proteoglycans, whereas there was a major band at 56 kDa in endo- β -galactosidase-digested monkey corneal stromal extracts (d) (Fig. 1D). Using this antibody, we found keratocan to be expressed by keratocytes and the extracellular matrix in the entire monkey corneal stroma, but not by the corneal epithelium (Fig. 1E) or the corneal endothelium (Fig. 1F). These results showed that rhesus monkey keratocytes had a dendritic morphology and extensive cell-cell contacts, and expressed both CD34 and keratocan, similar to that which we have reported in human keratocytes.^{7,15}

Morphologic Changes in Different Media

The monkey corneal stroma was subjected to collagenase digestion. The resultant cell suspension yielded approximately 1.5×10^5 cells per cornea. Within 24 hours after seeding on plastic, cells attached well in DMEM/ITS, DMEM/10% FBS, or KSF, but exhibited a distinctly different morphology. As reported for human keratocytes,^{7,15} cells cultured in DMEM/ITS for 7 days did not grow and showed a mixture of flattened and dendritic cells (Fig. 2A), whereas cells cultured in DMEM/10% FBS for 7 days reached confluence and showed a flattened fibroblastic morphology (Fig. 2B). In contrast, cells cultured in KSF for 7 days had a higher cell density than DMEM/ITS and maintained a dendritic morphology (Fig. 2C).

To determine whether the dendritic morphology of keratocytes could be similarly maintained in KSF in other species, we isolated primary cells from human (Fig. 2D), rabbit (Fig. 2E), and mouse (Fig. 2F) corneal stroma in the same manner and cultured them in KSF on plastic for 48 hours. We noted that all of them showed prominent dendritic processes and extensive intercellular contacts similar to that shown in monkey keratocytes (Fig. 2C). Both rabbit and human cells had a triangular cell body and longer dendrites; mouse cells had a rounder cell body and thinner dendrites. These data showed that the dendritic morphology could be similarly maintained in KSF for primary cultures of monkey, human, rabbit, and mouse keratocytes.

Cell Proliferation and Continuous Passages in KSF

To verify that cells were indeed proliferating in KSF, we performed the MTT assay at days 3 and 7 and immunostaining of Ki67 in primary cells at day 7. The number of cells measured by MTT did not change significantly when cells were cultured in DMEM/ITS, but increased significantly when cultured in DMEM/10% FBS from days 3 to 7 (Fig. 3A, $P < 0.01$). The number of cells in KSF estimated by MTT was between that of DMEM/ITS and DMEM/10% FBS (Fig. 3A; $P < 0.05$; between day 3 and day 7). When assayed by the proportion of positive Ki67 nuclei, cellular proliferation in KSF was also between that in DMEM/10% FBS and that in DMEM/ITS (Fig. 3B; $P < 0.05$, both between KSF and DMEM/10% FBS or DMEM/ITS). Cells maintained in DMEM/ITS could not be subcultured to passage 1. They immediately adopted a flattened morphology when subcultured in DMEM/10% FBS at passage 1 (Fig. 3C). In contrast, cells subcultured in KSF continued to maintain a dendritic morphology at passages 8 (Fig. 3D) and 15 (Fig. 3E). These results indicate that cells continued to maintain a dendritic morphology on plastic so long as they were cultured in KSF. They numbered approximately 2.0×10^5 in a 60-mm dish at each passage.

Modulation of Keratocyte Phenotype by Medium Switch

Because in vivo monkey keratocytes expressed keratocan and CD34 (Fig. 1), we examined whether keratocan and CD34 proteins were continuously expressed by dendritic cells that were maintained at late passages in KSF and whether such expression could be altered if the medium was switched to DMEM/ITS or DMEM/10% FBS. When passage-14 cells cultured in KSF were subcultured in DMEM/ITS or DMEM/10% FBS for 14 days, the dendritic morphology was changed to a flattened (fibroblastic) shape (Figs. 4A, 4B). In contrast, cells continuously subcultured in KSF maintained a dendritic morphology (Fig. 4C).

Immunostaining revealed that expression of keratocan was markedly attenuated in cells subcultured in DMEM/ITS (Fig. 4D) or DMEM/10% FBS (Fig. 4E), but continued in those cultured in KSFM (Fig. 4F). Similarly, expression of CD34 was markedly downregulated in cells subcultured in DMEM/ITS (Fig. 4G) or DMEM/10% FBS (Fig. 4H), but continued in those cultured in KSFM (Fig. 4I). Because ALDH is a marker of human keratocytes,⁴ we also found that ALDH was expressed in primary cells cultured in DMEM/ITS (Fig. 4J), lost in cells cultured in DMEM/10% FBS (Fig. 4K), but maintained in cells cultured in KSFM (Fig. 4L). Expression of ALDH was similarly downregulated when passage-14 keratocytes were subcultured in either DMEM/ITS or DMEM/10% FBS (not shown). These results indicated that the dendritic morphology of monkey keratocytes correlated well with expression of keratocan, CD34 and ALDH and such a phenotype could be maintained in KSFM, but lost when the medium was switched to either DMEM/10% FBS or DMEM/ITS.

Modulation of the Keratocyte Phenotype by Increasing Extracellular $[Ca^{2+}]$ and Adding FBS to KSFM

KSFM is culture medium supplemented by growth factors including epidermal growth factor (EGF) and bFGF, and differs from DMEM-base medium in many respects. The major features of KSFM are a low $[Ca^{2+}]$ and a lack of FBS. We thus wondered whether high $[Ca^{2+}]$ or addition of 10% FBS or a combination of both might modulate the keratocyte phenotype determined by expression of keratocan. To do so, we measured the promoter activities after transient transfection of Aden-track-Kerap3.2-intron-ECFP/BpA adenovirus containing the CMV-promoter-driven EGFP and keratocan promoter-driven ECFP in passage-1 cells. In a given cell, expression of EGFP reflects the background transfection while expression of ECFP reflects the keratocan promoter activity. We also monitored the protein expression of keratocan and CD34 by immunostaining.

Compared with the dendritic morphology of keratocytes cultured in KSFM (Fig. 5A), most cells remained dendritic, but some cells became flattened in KSFM when $[Ca^{2+}]$ was increased to 1.8 mM (Fig. 5B). In contrast, most of the cells lost the dendritic morphology and became flattened when 10% FBS was added in KSFM with low $[Ca^{2+}]$ (Fig. 5C) or with high $[Ca^{2+}]$ (Fig. 5D). The percentage of ECFP-expressing cells to EGFP-expressing cells of the control cultured in KSFM alone was $70.3 \pm 9.2\%$ (mean $\pm$ SD; Fig. 5E). That percentage decreased to $62.0 \pm 9.6\%$, $33.3 \pm 5.4\%$, and $29.8 \pm 4.5\%$ when cells were cultured in KSFM with 1.8 mM $[Ca^{2+}]$ (Fig. 5F), in KSFM with 10% FBS (Fig. 5G), and KSFM with 1.8 mM $[Ca^{2+}]$ and 10% FBS (Fig. 5H), respectively ($P < 0.01$ for KSFM versus KSFM+FBS or KSFM + $[Ca^{2+}]$ +FBS; Fig. 5U). There was no significant difference in those percentages between KSFM+ $[Ca^{2+}]$ +FBS and DMEM/10% FBS nor between KSFM and KSFM+ $[Ca^{2+}]$. Immunostaining showed expression of keratocan in cells cultured in KSFM (Fig. 5M) and in KSFM with high $[Ca^{2+}]$ (Fig. 5N), but expression was lost in KSFM with 10% FBS (Fig. 5O) and in KSFM with high $[Ca^{2+}]$ and 10% FBS (Fig. 5P). Expression of CD34 was observed in cells cultured in KSFM (Fig. 5Q) and KSFM with high $[Ca^{2+}]$ (Fig. 5R), but was lost in KSFM with 10% FBS (Fig. 5S) and in KSFM with high $[Ca^{2+}]$ and 10% FBS (Fig. 5T). These results indicate that the keratocyte phenotype was not significantly affected in KSFM by increasing $[Ca^{2+}]$, but was lost by addition of FBS. The latter detrimental effect was synergistic with increasing $[Ca^{2+}]$.

Suppression of TGF- β 1 and - β RII Promoter Activities

We then wondered whether TGF- β signaling was similarly modulated by increasing $[Ca^{2+}]$ or addition of 10% FBS, or a combination of both in KSFM, by measuring the promoter activity of TGF- β 1 and - β RII after transient adenoviral transfection. Compared with the control (i.e., cells cultured in DMEM/10% FBS and adjusted by background transfection with CMV- β -Gal), the promoter activity of both TGF- β 1 and - β RII was significantly decreased in cells cultured

in KSFM (Fig. 6, $P < 0.05$). There was no significant difference in the promoter activity between KSFM and DMEM/ITS (Fig. 6). Compared with the control cultured in KSFM alone, increased $[Ca^{2+}]$ or addition of 10% FBS did not change the promoter activity for both TGF- β 1 and - β RII (Fig. 6; $P > 0.05$). In contrast, a combination of increased $[Ca^{2+}]$ and addition of 10% FBS significantly upregulated the promoter activity for TGF- β 1 and - β RII (Fig. 6, $P < 0.05$ and $P < 0.01$, respectively). These results further support the notion that the loss of keratocyte phenotype with respect to dendritic morphology and expression of keratocan and CD34 as a result of increased $[Ca^{2+}]$ and addition of 10% FBS correlates with upregulation of the transcriptional activity of the TGF- β 1 and - β RII genes.

Suppression of Smad-Mediated TGF- β Signaling

To determine whether the aforementioned phenotype changes and suppression of transcription of TGF- β 1 and TGF- β RII genes correlates with change of Smad-mediated signaling, we performed immunostaining for Smad2 and Smad4. Most cells cultured in DMEM/ITS or KSFM showed cytoplasmic localization of Smad2 and Smad4, whereas most cells cultured in DMEM/10% FBS or KSFM with increased $[Ca^{2+}]$ and addition of 10% FBS showed nuclear localization of Smad2 and Smad4 (Fig. 7). The percentage of cells exhibiting nuclear accumulation of Smad2, an index suggestive of phosphorylation of Smad2, was $38\% \pm 7.6\%$ (mean $\pm$ SD) in DMEM/ITS and $88.7\% \pm 4.0\%$ in DMEM/10% FBS, of which both were significantly higher than $19.3\% \pm 5.1\%$ in KSFM (Fig. 7, both, $**P < 0.01$). Even when 4 ng/mL TGF- β 1 was added in KSFM for 48 hours, the percentage of nuclear accumulation of Smad2 in cells increased to $34.7\% \pm 4.9\%$, which was still not higher than that of DMEM/ITS (Fig. 7, $P > 0.05$). Similarly, the percentage of nuclear accumulation of Smad4 was $27.7\% \pm 1.5\%$, $90.7\% \pm 2.1\%$, and $12.0\% \pm 3.0\%$ in DMEM/ITS, DMEM/10% FBS, and KSFM, respectively (Fig. 7). These results indicated that Smad-mediated TGF- β signaling was significantly downregulated in cells cultured in KSFM.

Discussion

Earlier studies used the dendritic morphology and extensive intercellular contacts as the hallmark for the keratocyte phenotype.¹² Subsequently, other markers for keratocytes have been reported, including keratan sulfate-containing proteoglycans,^{3,4,34} such as keratocan,^{4,15,35} CD34,^{5,7} and ALDH.^{4,36} In this study, we noted that similar to in vivo human keratocytes,¹⁵ in vivo primate keratocytes exhibited a dendritic morphology and expressed both keratocan and CD34 (Fig. 1). We further provided strong experimental evidence supporting that the dendritic morphology and expression of keratocan, CD34, and ALDH can be maintained in rhesus monkey keratocytes on plastic if cultured in a serum-free KSFM. This accomplishment represents a significant advance in the field of keratocyte biology, because all previous attempts in other media have failed. In plastic cultures, expression of one or several of the aforementioned markers has been reported for bovine keratocytes using serum-free DMEM¹² or DMEM/F12,³⁷ for rabbit keratocytes using serum-free DMEM,³⁸ and for human keratocytes using serum-free DMEM/ITS.¹⁵ Nevertheless, keratocytes cultured in these media cease proliferation, a phenomenon also found in monkey keratocytes cultured in DMEM/ITS (Fig. 3). Similar to DMEM/ITS, another serum-free medium called KSFM, also maintained on plastic, cultures the dendritic morphology of keratocytes from several species, including human, rabbit, mouse, and monkey (Fig. 2). In KSFM, human dendritic keratocytes expressed keratocan (not shown) and monkey keratocytes expressed keratocan, CD34, and ALDH (Figs. 4, 5). However, unlike DMEM/ITS, in which cells ceased proliferation, keratocytes cultured in KSFM continued to proliferate and could be subcultured until passage 15 before senescence (Fig. 3). During this period, they continued to maintain a dendritic morphology and expressed keratocan, CD34, and ALDH (Fig. 4). This culture medium thus allows us to expand

keratocytes while maintaining their phenotype from several species, to investigate how they may control corneal transparency in the future.

Although the growth potential can be promoted in DMEM by adding FBS, bovine¹² and rabbit¹³ keratocytes rapidly lose the dendritic morphology and the expression of keratan sulfate-containing keratocan,¹⁵ and CD34,⁷ in human keratocytes, and the expression of ALDH in bovine keratocytes.⁴ In this study, we also noted that the dendritic morphology rapidly turned to a flattened fibroblastic morphology if freshly isolated monkey keratocytes were directly seeded in DMEM/10% FBS (Fig. 2) or if primary (Fig. 3) or passage-14 (Fig. 4) keratocytes maintained in KSFM were subcultured in DMEM/10% FBS. Although cell proliferation was promoted in DMEM/10% FBS (Fig. 3), these cells lost expression of keratocan, CD34, and ALDH (Fig. 4). These findings, consistent with others described herein, further revealed that DMEM/10% FBS exerted a universal detrimental effect on the maintenance of the keratocyte phenotype.

The presence of FBS is a major difference between DMEM/10% FBS and KSFM. Addition of 10% FBS in KSFM caused monkey keratocytes to lose the dendritic morphology and the expression of keratocan and CD34, and to downregulate the keratocan promoter activity (Fig. 5). FBS contains TGF- β _{20,39} and vitamin A⁴⁰ both of which can induce autocrine and paracrine production of TGF- β ,^{41,42} which promotes myofibroblast differentiation.^{4,14,21,22} Herein, we note that the promoter activities of TGF- β 1 and - β RII were not significantly upregulated when 10% FBS was added to KSFM compared with DMEM/10% FBS (Fig. 6) although the keratocyte phenotype measured by expression of keratocan and CD34 and by keratocan promoter activities was already lost (Fig. 5). These results suggest that FBS led to the loss of the keratocytes phenotype without transcriptional upregulation of TGF- β and that the extent of fibroblast differentiation stimulated by 10% FBS was more advanced in DMEM than in KSFM.

Such a difference may be attributable to $[Ca^{2+}]$, which is present at 0.07 mM in KSFM, much lower than the 1.8 and 0.9 mM in DMEM and DMEM/F12, respectively. Extracellular $[Ca^{2+}]$ can affect the balance between proliferation and differentiation in many cell types. For example, the growth of cultured dermal fibroblasts is inhibited at low but promoted in high extracellular $[Ca^{2+}]$.⁴³ An increase of extracellular $[Ca^{2+}]$ stimulates DNA synthesis of 3T3 fibroblasts by activating mitogen activated protein (MAP) kinase.⁴³ Furthermore, the growth potential is promoted by high extracellular $[Ca^{2+}]$ (>1.0 mM), even without growth factors.⁴³ A medium containing a high $[Ca^{2+}]$ concentration has been used to culture fibroblasts, because it can prevent apoptosis and promote their growth by activating MAP kinase.⁴⁴ It remains unknown whether keratocytes (i.e., neural crest-derived mesenchymal cells) prefer a high- $[Ca^{2+}]$ medium in a similar manner to fibroblasts. In our study, we noted that an increase of $[Ca^{2+}]$ to 1.8 mM alone in KSFM was not sufficient to alter the dendritic morphology and expression of keratocan and CD34 (Fig. 5), nor did it upregulate the keratocan promoter activity and the promoter activities of TGF- β 1 and - β RII (Figs 5· 6). However, an increase of $[Ca^{2+}]$ to 1.8 mM in KSFM with simultaneous addition of 10% FBS synergistically upregulated the promoter activities of TGF- β 1 and - β RII, and nuclear translocation of Smad2 and Smad4 to the same extent as did DMEM/10% FBS (Figs. 6· 7). These results indicate that high $[Ca^{2+}]$ and FBS synergistically upregulates Smad-mediated TGF- β signaling. In other words, FBS's detrimental effect against the keratocyte phenotype is contingent on high $[Ca^{2+}]$. This finding indicates that keratocytes responded to $[Ca^{2+}]$ similar to epidermal keratinocytes, in which TGF- β stimulates differentiation in high $[Ca^{2+}]$,^{45–48} but has no effect or inhibits differentiation in low $[Ca^{2+}]$.^{45,46,49} In epidermal keratinocytes, the synergism between high $[Ca^{2+}]$ and TGF- β action can further be explained by the fact that TGF- β mRNA can be induced by high $[Ca^{2+}]$.⁴¹ In fibroblasts, TGF- β signaling depends on extracellular $[Ca^{2+}]$.²⁶ These

data collectively indicate that a low- $[Ca^{2+}]$ medium such as in KSFM plays a major role in downregulating TGF- β signaling.

Monkey keratocytes continuously expanded in KSFM lost their normal phenotype, as defined by the expression of keratocan, CD34 and ALDH when the medium was switched to DMEM/ITS (Fig. 4). Because a mere increase of $[Ca^{2+}]$ alone in KSFM was not sufficient to alter such a phenotype (Fig. 5), we thus speculate that withdrawal of growth factor supplement from KSFM (when switched to DMEM/ITS) is responsible for such a phenotypic change. If indeed our interpretation were correct that the keratocyte phenotype depends on suppression of TGF- β signaling, withdrawal of growth factor supplement should be sufficient to activate TGF- β signaling. It is well known that the final outcome of transactivation of targeted genes mediated by TGF- β signaling can be modified by other signaling pathways mediated by MAP kinase, JNK, and Akt that may interplay and crosstalk with Smad-mediated signaling (for review, see Refs. 50-51). Future studies are needed to delineate how each growth factor in KSFM participates in the inception of Smad-mediated signaling, resulting in suppression of TGF- β signaling, as another mechanism explaining why a serum-free, low- $[Ca^{2+}]$ medium can maintain the keratocyte phenotype while promoting cell proliferation.

References

1. Ueda A, Nishida T, Otori T, Fujita H. Electron-microscopic studies on the presence of gap junctions between corneal fibroblasts in rabbits. *Cell Tissue Res* 1987;249:473–475. [PubMed: 3621310]
2. Poole CA, Brookes NH, Clover GM. Keratocyte networks visualised in the living cornea using vital dyes. *J Cell Sci* 1993;106:685–692. [PubMed: 8282773]
3. Funderburgh JL, Funderburgh ML, Mann MM, et al. Synthesis of corneal keratan sulfate proteoglycans by bovine keratocytes in vitro. *J Biol Chem* 1996;271:31431–31436. [PubMed: 8940154]
4. Funderburgh JL, Mann MM, Funderburgh ML. Keratocyte phenotype mediates proteoglycan structure: a role for fibroblasts in corneal fibrosis. *J Biol Chem* 2003;278:45629–45637. [PubMed: 12933807]
5. Toti P, Tosi GM, Traversi C, et al. CD-34 stromal expression pattern in normal and altered human corneas. *Ophthalmology* 2002;109:1167–1171. [PubMed: 12045061]
6. Joseph A, Hossain P, Jham S, et al. Expression of CD34 and L-selectin on human corneal keratocytes. *Invest Ophthalmol Vis Sci* 2003;44:4689–4692. [PubMed: 14578387]
7. Espana EM, Kawakita T, Liu CY, Tseng SC. CD-34 expression by cultured human keratocytes is downregulated during myofibroblast differentiation induced by TGF-beta1. *Invest Ophthalmol Vis Sci* 2004;45:2985–2991. [PubMed: 15326111]
8. SundarRaj N, Fite D, Belak R, et al. Proteoglycan distribution during healing of corneal stromal wounds in chick. *Exp Eye Res* 1998;67:433–442. [PubMed: 9820791]
9. Carlson EC, Wang IJ, Liu CY, et al. Altered KSPG expression by keratocytes following corneal injury. *Mol Vis* 2003;9:615–623. [PubMed: 14654769]
10. Jester JV, Petroll WM, Barry PA, Cavanagh HD. Expression of alpha-smooth muscle (alpha-SM) actin during corneal stromal wound healing. *Invest Ophthalmol Vis Sci* 1995;36:809–819. [PubMed: 7706029]
11. Jester JV, Petroll WM, Cavanagh HD. Corneal stromal wound healing in refractive surgery: the role of myofibroblasts. *Prog Retin Eye Res* 1999;18:311–356. [PubMed: 10192516]
12. Beals MP, Funderburgh JL, Jester JV, Hassell JR. Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture. *Invest Ophthalmol Vis Sci* 1999;40:1658–1663. [PubMed: 10393032]
13. Jester JV, Barry-Lane PA, Cavanagh HD, Petroll WM. Induction of alpha-smooth muscle actin expression and myofibroblast transformation in cultured corneal keratocytes. *Cornea* 1996;15:505–516. [PubMed: 8862928]
14. Jester JV, Ho-Chang J. Modulation of cultured corneal keratocyte phenotype by growth factors/cytokines control in vitro contractility and extracellular matrix contraction. *Exp Eye Res* 2003;77:581–592. [PubMed: 14550400]

15. Espana EM, He H, Kawakita T, et al. Human keratocytes cultured on amniotic membrane stroma preserve morphology and express keratocan. *Invest Ophthalmol Vis Sci* 2003;44:5136–5141. [PubMed: 14638709]
16. Dahl IM, Johnsen W, Anseth A, Prydz H. The synthesis of glycosaminoglycans by corneal stroma cells in culture. *Exp Cell Res* 1974;88:193–197. [PubMed: 4278995]
17. Dahl IM, Coster L. Proteoglycan biosynthesis in cultures of corneas and corneal stroma cells from adult rabbits. *Exp Eye Res* 1978;27:175–190. [PubMed: 680033]
18. Dahl IM. Biosynthesis of proteoglycans and hyaluronate in rabbit corneal fibroblast cultures: variation with age of the cell line and effect of foetal calf serum. *Exp Eye Res* 1981;32:419–433. [PubMed: 7238627]
19. Hassell JR, Schrecengost PK, Rada JA, et al. Biosynthesis of stromal matrix proteoglycans and basement membrane components by human corneal fibroblasts. *Invest Ophthalmol Vis Sci* 1992;33:547–557. [PubMed: 1544783]
20. Kropf J, Schurek JO, Wollner A, Gressner AM. Immunological measurement of transforming growth factor-beta 1 (TGF-beta1) in blood: assay development and comparison. *Clin Chem* 1997;43:1965–1974. [PubMed: 9342020]
21. Petridou S, Masur SK. Immunodetection of connexins and cadherins in corneal fibroblasts and myofibroblasts. *Invest Ophthalmol Vis Sci* 1996;37:1740–1748. [PubMed: 8759341]
22. Funderburgh JL, Funderburgh ML, Mann MM, et al. Proteoglycan expression during transforming growth factor beta-induced keratocyte-myofibroblast transdifferentiation. *J Biol Chem* 2001;276:44173–44178. [PubMed: 11555658]
23. Tseng SC, Li DQ, Ma X. Suppression of transforming growth factor-beta isoforms, TGF-beta receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. *J Cell Physiol* 1999;179:325–335. [PubMed: 10228951]
24. Lee SB, Li DQ, Tan DT, et al. Suppression of TGF-beta signaling in both normal conjunctival fibroblasts and pterygial body fibroblasts by amniotic membrane. *Curr Eye Res* 2000;20:325–334. [PubMed: 10806447]
25. Martineau I, Lacoste E, Gagnon G. Effects of calcium and thrombin on growth factor release from platelet concentrates: kinetics and regulation of endothelial cell proliferation. *Biomaterials* 2004;25:4489–4502. [PubMed: 15046940]
26. Gooch JL, Gorin Y, Zhang BX, Abboud HE. Involvement of calcineurin in transforming growth factor-beta-mediated regulation of extracellular matrix accumulation. *J Biol Chem* 2004;279:15561–15570. [PubMed: 14742441]
27. Romero JR, Rivera A, Lanca V, et al. Na⁺/Ca²⁺ Exchanger activity modulates connective tissue growth factor mRNA expression in transforming growth factor β 1- and Des-Arg10-kallidin-stimulated myofibroblasts. *J Biol Chem* 2005;280:14378–14384. [PubMed: 15703175]
28. Liu C, Arar H, Kao C, Kao WW. Identification of a 3.2 kb 5'-flanking region of the murine keratocan gene that directs beta-galactosidase expression in the adult corneal stroma of transgenic mice. *Gene* 2000;250:85–96. [PubMed: 10854782]
29. Chen J, Huber BT, Grand RJ, Li W. Recombinant adenovirus coexpressing covalent peptide/MHC class II complex and B7-1: in vitro and in vivo activation of myelin basic protein-specific T cells. *J Immunol* 2001;167:1297–1305. [PubMed: 11466346]
30. He TC, Zhou S, da Costa LT, et al. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci USA* 1998;95:2509–2514. [PubMed: 9482916]
31. Kawakita T, Espana EM, He H, et al. Keratocan expression of murine keratocytes is maintained on amniotic membrane by downregulating transforming growth factor-beta signaling. *J Biol Chem* 2005;280:27085–27092. [PubMed: 15908433]
32. Bae HW, Geiser AG, Kim DH, et al. Characterization of the promoter region of the human transforming growth factor-beta type II receptor gene. *J Biol Chem* 1995;270:29460–29468. [PubMed: 7493985]
33. Roth GS, Mattison JA, Ottinger MA, et al. Aging in rhesus monkeys: relevance to human health interventions. *Science* 2004;305:1423–1426. [PubMed: 15353793]

34. Berryhill BL, Kader R, Kane B, et al. Partial restoration of the keratocyte phenotype to bovine keratocytes made fibroblastic by serum. *Invest Ophthalmol Vis Sci* 2002;43:3416–3421. [PubMed: 12407151]
35. Liu C-Y, Siraishi A, Kao CW-C, et al. The cloning of mouse keratocan cDNA and genomic DNA and the characterization of its expression during eye development. *J Biol Chem* 1999;273:22584–22588. [PubMed: 9712886]
36. Jester JV, Møller-Pedersen T, Huang J, et al. The cellular basis of corneal transparency: evidence for ‘corneal crystallins’. *J Cell Sci* 1999;112:613–622. [PubMed: 9973596]
37. Berryhill BL, Beales MP, Hassell JR. Production of prostaglandin D synthase as a keratan sulfate proteoglycan by cultured bovine keratocytes. *Invest Ophthalmol Vis Sci* 2001;42:1201–1207. [PubMed: 11328728]
38. Jester JV, Huang J, Petroll WM, Cavanagh HD. TGFbeta induced myofibroblast differentiation of rabbit keratocytes requires synergistic TGFbeta, PDGF and integrin signaling. *Exp Eye Res* 2002;75:645–657. [PubMed: 12470966]
39. Masui T, Wakefield LM, Lechner JF, et al. Type beta transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. *Proc Natl Acad Sci USA* 1986;83:2438–2442. [PubMed: 2871553]
40. Fuchs E, Green H. Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. *Cell* 1981;25:617–625. [PubMed: 6169442]
41. Glick AB, Flanders KC, Danielpour D, et al. Retinoic acid induces transforming growth factor-beta 2 in cultured keratinocytes and mouse epidermis. *Cell Regul* 1989;1:87–97. [PubMed: 2519621]
42. Bascom CC, Wolfshohl JR, Coffey RJ Jr, et al. Complex regulation of transforming growth factor beta 1, beta 2, and beta 3 mRNA expression in mouse fibroblasts and keratinocytes by transforming growth factors beta 1 and beta 2. *Mol Cell Biol* 1989;9:5508–5515. [PubMed: 2586525]
43. Huang S, Maher VM, McCormick JJ. Extracellular Ca²⁺ stimulates the activation of mitogen-activated protein kinase and cell growth in human fibroblasts. *Biochem J* 1995;310:881–885. [PubMed: 7575422]
44. Toda S, Yokoi F, Yamada S, et al. Air exposure promotes fibroblast growth with increased expression of mitogen-activated protein kinase cascade. *Biochem Biophys Res Commun* 2000;270:961–966. [PubMed: 10772933]
45. Reiss M, Sartorelli AC. Regulation of growth and differentiation of human keratinocytes by type beta transforming growth factor and epidermal growth factor. *Cancer Res* 1987;47:6705–6709. [PubMed: 2445478]
46. Coffey RJ Jr, Sipes NJ, Bascom CC, et al. Growth modulation of mouse keratinocytes by transforming growth factors. *Cancer Res* 1988;48:1596–1602. [PubMed: 2449957]
47. Wilke MS, Hsu BM, Wille JJ Jr, et al. Biologic mechanisms for the regulation of normal human keratinocyte proliferation and differentiation. *Am J Pathol* 1988;131:171–181. [PubMed: 2451428]
48. Matsumoto K, Hashimoto K, Hashiro M, et al. Modulation of growth and differentiation in normal human keratinocytes by transforming growth factor-beta. *J Cell Physiol* 1990;145:95–101. [PubMed: 2211846]
49. Reiss M, Zhou ZL. Uncoupling of the calcium-induced terminal differentiation and the activation of membrane-associated transglutaminase in murine keratinocytes by type-beta transforming growth factor. *Exp Cell Res* 1989;183:101–111. [PubMed: 2472281]
50. Massague J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J* 2000;19:1745–1754. [PubMed: 10775259]
51. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577–584. [PubMed: 14534577]

Figure 1.

Characterization of rhesus monkey keratocytes in vivo. Immediately after adding collagenase solution to the rhesus monkey cornea, both phase-contrast microscopy (A) and a cell-viability assay (B) showed a characteristic dendritic morphology with extensive cell-cell contacts in a three-dimensional manner. CD34 was expressed in the cytoplasm of keratocytes by immunohistochemistry (C) and immunofluorescence staining (C, *inset*). The antibody raised against human keratocan cross-reacted with monkey keratocan by Western blot analysis; a blot in a high-molecular-weight region was found in undigested samples (u), whereas a major band at 56 kDa was found in endo- β -galactosidase-digested cornea (d). (D) Use of this antibody showed that keratocan (*green*) is expressed in keratocytes and also distributed throughout the stroma, but not in the corneal epithelium (E) or endothelium (F). *Red*: nuclei (PI). Bar, 50 μ m.

Figure 2.

Morphologic differences. Primary cells cultured in DMEM/ITS for 7 days showed a mixture of flattened and dendritic cells (**A**). In contrast, cells cultured in DMEM/10% FBS for 7 days reached confluence and showed a flattened fibroblastic morphology (**B**). Primary cells cultured in KSFM for 7 days had a higher cell density than those in DMEM/ITS, and showed a dendritic morphology (**C**). The dendritic morphology was also maintained in primary cultures of human (**D**), rabbit (**E**), and mouse (**F**) keratocytes in KSFM. Bar, 50 μ m.

Figure 3.

Cellular proliferation in KSFM. When measured by MTT assay (**A**) at days 3 and 7 of primary cultures, cellular proliferation did not change significantly in DMEM/ITS, but increased markedly in DMEM/10% FBS ($P < 0.01$). The cellular proliferation of KSFM cultures was between that of DMEM/10% FBS and that of DMEM/ITS ($P < 0.05$ between days 3 and 7). When assayed by the percentage of positive Ki67 nuclei in primary cultures at day 7 (**B**), cell proliferation in KSFM was significantly lower than DMEM/10% FBS ($*P < 0.05$). Error bars, SD. Keratocytes maintained in DMEM/10% FBS immediately adopted a flattened morphology when subcultured at passage 1 (**C**). In contrast, cells in KSFM continued to maintain a dendritic morphology with extensive cell–cell contacts, even when subcultured to passages 8 (**D**) and 15 (**E**). Bar, 100 μm .

Figure 4.

Keratocyte phenotype after a switch of medium. Cells continuously expanded in KSFM up to passage 14 were subcultured in KSFM, DMEM/ITS, and DMEM/10% FBS for 14 days for a comparison. Cells became flattened (fibroblastic) when switched to either DMEM/ITS (**A**) or DMEM/10% FBS (**B**). In contrast, cells cultured in KSFM continued to maintain a dendritic morphology (**C**). Furthermore, expression of keratocan was markedly attenuated in DMEM/ITS (**D**) or DMEM/10% FBS (**E**) compared with that of KSFM (**F**). Expression of CD34 was also markedly attenuated when cultured in DMEM/ITS (**G**) or DMEM/10% FBS (**H**), but was maintained in KSFM (**I**). ALDH was expressed in primary cells in DMEM/ITS (**J**) and in KSFM (**L**), but not in DMEM/10% FBS (**K**). Bars, 50 μ m.

Figure 5.

Keratocan promoter activity was maintained in KSFM. Aden-track-Kerapr3.2-intron-ECFP/BpA adenovirus (200 MOI) was transfected into rhesus monkey keratocytes (passage 1) for 24 hours in KSFM (A), 1.8 mM KSFM+ [Ca²⁺] (B), KSFM+10% FBS (C), or KSFM+ [Ca²⁺]+FBS (D). Expression of EGFP (monitoring background transfection) showed that the transfection efficiency was >80% in all conditions (E–H). ECFP expression (monitoring keratocan promoter activity) was observed in all conditions, but the percentage of ECFP to EGFP was 70.3% ± 9.2%, 62.0% ± 9.6%, 33.3% ± 5.4%, and 29.8% ± 4.5% (mean ± SD) in KSFM (I), 1.8 mM KSFM+ [Ca²⁺] (J), KSFM+10% FBS (K), and KSFM+ [Ca²⁺]+FBS (L), respectively (***P* < 0.01). Immuno-staining against keratocan and CD34 showed cells clearly expressed in KSFM (M, Q) and KSFM+ [Ca²⁺] (N, R), but lost in KSFM+FBS (O, S) and SFM+[Ca²⁺]+FBS (P, T). Keratocan promoter activity (number of ECFP-positive cells

divided by number of EGFP-positive cells of each culturing condition is demonstrated in (U).
** $P < 0.01$. Bar, 50 μm .

Figure 6.

TGF- β 1 and - β RII promoter activities. TGF- β 1 and - β RII transcriptional activity of rhesus monkey keratocytes was compared in DMEM/ITS, DMEM/10% FBS, and KSFM. Passage-1 cells were cotransfected with plasmids containing TGF- β 1 or TGF- β RII promoter-luc or CMV- β -galactosidase for 48 hours. Cell extracts were assayed for activities of both luciferase and β -galactosidase. The relative luciferase promoter activity of both TGF- β 1 and - β RII was suppressed significantly in cells cultured in KSFM compared with DMEM/10% FBS (* $P < 0.05$). To analyze further the effects of $[Ca^{2+}]$ and FBS, four different culture media were compared: KSFM, KSFM+additional $[Ca^{2+}]$ (1.8 mM), KSFM+FBS (10% vol/vol), and KSFM+both $[Ca^{2+}]$ and FBS. The promoter activity of TGF- β 1 or - β RII was suppressed in cells cultured in KSFM (* $P < 0.05$), and were both promoted in cells cultured in KSFM by $[Ca^{2+}]$ and FBS, synergistically (TGF- β 1, * $P < 0.05$; TGF- β RII, ** $P < 0.01$).

Figure 7.

Smad-mediated TGF- β signaling. To monitor the Smad-mediated TGF- β signaling, we performed immunostaining of Smad2 and Smad4. Smad2 and Smad4 were expressed diffusely and predominantly in the cytoplasm in DMEM/ITS or KSFM. In contrast, they were predominantly perinuclear and nuclear in DMEM/FBS or KSFM with additional [Ca²⁺] and FBS. Nuclear accumulation of Smad2 and Smad4 was significantly promoted in DMEM/FBS and KSFM+[Ca²⁺]+FBS (both $**P < 0.01$). Bar, 50 μ m.