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MK/T-1, an immortalized fibroblast cell line derived using cultures of mouse corneal stroma

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Purpose: Immortalized cell lines representing fibroblast cells from corneal stroma would facilitate studies of corneal cell biology and injury response.
Methods: Primary cultures of cells derived from mouse corneal stroma were transfected with a human telomerase reverse transcriptase (hTERT) expression construct to maximize chances of cellular immortalization. A resulting cell line was analyzed for telomerase activity, cell growth characteristics, senescence and gene expression patterns. Specific responses to transforming growth factor β (TGF-β) were also analyzed.
Results: An immortalized cell line was derived and was named MK/T-1. MK/T-1 cells show no signs of cellular senescence or transformation at over 100 passages. Telomerase activity was significantly higher in MK/T-1 cells as compared to the parental cell cultures. However, relative telomere length (RTL) in the MK/T-1 and parental cells was not significantly different. Senescence associated β-galactosidase (SA-β-Gal) activity was not detected in late passage MK/T-1 cells while the parental cells had already upregulated SA-β-Gal at high levels by passage 9. The MK/T-1 cells express vimentin, tubulin, lumican, mimecan, decorin and collagen I, but not keratocan. Exposure of the MK/T-1 cells to TGF-β induces the expression of smooth muscle α-actin (ASMA), the activation of MAP Kinase (p38-MAPK) and morphological changes consistent with cytoskeletal reorganization.
Conclusions: MK/T-1 cells represent an immortalized fibroblast cell line derived using cultures from corneal stroma cell preparations. Expression of hTERT may contribute to immortalization of the MK/T-1 cells by a mechanism other than increases in RTL. MK/T-1 cells may be a useful model in which to study the responses of corneal fibroblast cells to cytokines and other diverse environmental factors in vitro.

Keratocytes, the cells composing the normal corneal stroma, are responsible for maintaining corneal clarity [1-6]. Corneal damage as a result of a range of diseases (keratoconus) or injury (wounding, surgical intervention) can involve cellular changes in keratocytes and can lead to vision loss and/or blindness [7-19]. The specific regulatory pathways which are responsible for maintaining homeostasis and signaling changes in cell fate during disease and injury in corneal cells are at present only partially characterized. The unique profile of extracellular matrix proteoglycans found in the corneal stroma and produced by keratocytes, including lumican, decorin, mimecan and keratocan may play important roles in the highly specialized microenvironment maintaining corneal transparency [4-6]. The derivation of permanent immortalized in vitro cell lines from normal corneal stroma would facilitate the study of the growth, differentiation and injury response of the cornea. Although methods for the primary culture and preservation of keratocytes have been described [20-22], the only immortalized, permanent cell lines representing corneal stromal cells available are those generated through expression of transforming oncogenes [23]. A further hurdle is that what has been described previously as the true keratocyte phenotype can only be achieved in serum-free media, resulting in cellular quiescence [22]. Since cell lines must proliferate and therefore cannot be derived from quiescent cell populations, we surmised that proliferating cells derived from cultures prepared from corneal stroma may be immortalizable and may retain some characteristics consistent with fibroblast cells. Such a permanent cell line immortalized without transforming oncogenes might thus offer a permanent, stable and more unconstrained alternative in vitro system for studies of corneal fibroblast cell biology and biochemistry.

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METHODS

Animals: All mouse tissues used in this study were obtained from either sacrificed mice or from archives of paraffin embedded tissues prepared from wild type C57/B6 mice using procedures adherent to the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals).

Corneal cell cultures: To establish cultures from corneal stroma, epithelium and endothelium were removed from excised mouse corneas as previously described [24]. The stromas were diced into small pieces and treated with 0.05% Trypsin-EDTA solution (type XI, Sigma, St. Louis, MO), in phosphate balanced saline [PBS, 8 g/l NaCl, 0.4 g/l KCl, 1 g/l Glucose, 0.4 g/l $NaHCO_3$, 0.22 g/l Na_2EDTA (0.6 mM), pH 7.0] at 37 °C for 30 min. The matrix free stromal cells were collected by centrifugation and were cultured in DMEM plus 10% fetal bovine serum.

Immortalization: In an effort to derive a permanent immortalized cell line, we transfected a human telomerase reverse transcriptase subunit expression construct driven by the cytomegalovirus (CMV) promoter and containing the hygromycin resistance gene (pGRN; a generous gift from Geron Inc., Menlo Park, CA) into cultures of the corneal stroma derived cells between passage 2-5 using lipofectin reagent (Gibco, Rockville, MD). The resulting bulk transfected culture was expanded in the selective drug hygromycin (50 μg/ml) until confluent, trypsinized, plated to achieve dispersion of single cells and subsequently used to subclone individual cell lines using glass cloning cylinders (Corning, Cambridge, MA). Subcloned cell lines were expanded further in selective drug for analysis. One subclone retaining a morphology similar to the parental cells and showing contact inhibition was named MK/T-1 and was further studied. For routine passaging of the MK/T-1 cells, cultures at 60-80% of total confluency were trypsinized and replated into fresh tissue culture dishes at a 1/5 dilution approximately every 3 days.

Anchorage independent growth analysis: For growth in soft agar, 5 x 10^5, 2.5 x 10^5 and 1.25 x 10^5 SV40 Large T transformed IEM cells [25] or the same numbers of late passage pGRN-transfected MK/T-1 cells were plated into 60 mm dishes in 2 ml of 0.33% Agar Noble (Difco Laboratories, Detroit, MI) in complete culture media over a layer of 2 ml of 0.5% Agar Noble in complete media. Between 7 to 10 days after plating, total numbers of colonies growing in soft agar were counted.

Cytokine stimulation: For studies of the effects of TGF-β on expression of differentiation markers such as ASMA in MK/T-1 cells, the cells were treated with TGF-β2 at various concentrations for the times indicated below or left untreated for controls. Cultures prepared on glass coverslips for immunocytochemistry were rested overnight in medium containing 1% serum and stimulated the next day with 10 ng/ml TGF-β2 for 24 h. Coverslips were then fixed in 4% paraformaldehyde and stained in situ by fluorescence immunocytochemistry.

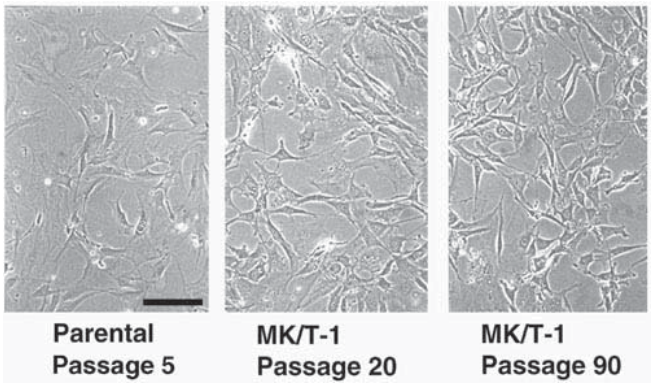

Figure 1. MK/T-1 cell morphology. Shown are phase contrast views of early (20 passage) and late (90 passage) MK/T-1 cells growing in a tissue culture dish compared to the parental untransfected cells derived from corneal stroma cultures at passage 5. MK/T-1 cells appear fibroblastic. Scale bar represents 50 μm.

Cells from separate cultures were harvested for preparation of whole cell lysates for western blotting as described below. For determination of expression of activated p38-MAPK, MK/T-1 cells were plated in DMEM supplemented with 10% FBS. After adhesion, cells were rested overnight in DMEM containing 1% FBS. Cells were then stimulated with TGF-β2 (5 ng/ml) for 1 h or left untreated.

TRAP assay: Telomerase activity was measured utilizing the telomere repeat amplification protocol (TRAP) with the Telomerase PCR ELISA kit (Boehringer Mannheim, Indianapolis, IN). The untransfected parental cells and the MK/T-1 cell line were harvested separately and cell numbers were counted using a hemacytometer. An equal number of cells from each cell preparation was used for preparing protein extracts for each TRAP reaction. The protein was extracted from the cell cultures and used in telomerase mediated elongation, adding the telomeric repeats to the 3' end of the biotin-labeled synthetic P1-TS-primer. This elongation step allows the P1-TS and P2 primers to generate PCR products with the telomerase specific 6 nucleotide increments. PCR amplification was performed using a DNA Thermal Cycler 480 (Perkin Elmer, Foster City, CA) following the programs suggested by Boehringer Mannheim methodology. The PCR products were resolved on a 12% polyacrylamide non-denaturing gel. The gel was electroblotted onto a positively charged nylon membrane. The membrane was then probed with a Streptavidin-AP conjugate (DuPont/NEN, Cambridge, MA) and developed with CDP-StarNucleic Acid Chemiluminescense Reagent (DuPont/NEN).

Relative telomere length: For determination of relative telomere length (RTL), equal quantities of genomic DNA isolated from early passage parental cultures and late passage MK/T-1 cells was digested with the *Sau3a I* restriction enzyme and subjected to electrophoresis in a 0.7% agarose gel for 18-24 h. The gel was then dried, denatured, washed in neutralizing buffer, prehybridized for 1 h at 37 °C and finally hybridized with a ^{32}P kinase-labeled oligonucleotide probe specific for telomere repeats (5'-TTA GGG TTA GGG TTA GGG-3') overnight at 37 °C. After washing the hybridized gel in 3 changes of 0.1% SSC at room temperature, the gel was mounted on filter paper and exposed to X ray film.

Senescence analysis: Senescence of the MK/T-1 cell line and the parental cells was compared by histochemical staining of both cell types for expression of senescence associated β-galactosidase (SA-β-Gal) activity [26]. Cells were cultured in DMEM + 10% FBS. The cells were trypsinized, counted and plated at the same cell numbers and incubated for six h. The media was then removed and the cells were gently rinsed one time with PBS. After removing the PBS, fixative (0.2% gluteraldehyde and 2% formaldehyde) was carefully added to the culture plate so as not to disturb the cells. The cells were incubated in fixative at room temperature for five min. The fixative was then slowly removed and the cells were rinsed in PBS three times. The stain solution [β-Gal Staining kit (Roche, Indianapolis, IN)], adjusted to pH 6.0 (for SA-β-Gal [26]), was added to each culture plate and incubated for 16 h at 37 °C. The plates were then removed from the incubator, stain-

ing substrate was removed from the plates and the plates were rinsed with PBS. Plates were then coverslipped for viewing and photography.

Northern analysis: Northern blot analysis was performed for detection of lumican, mimecan, decorin, keratocan and collagen α1 (I) chain. Glyceraldehyde phosphate dehydrogenase (gapdh) was used as an internal positive loading control. Total RNA was isolated from the cultured cells with TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) using procedures recommended by the manufacturer. Northern blotting was performed as described previously [27-29].

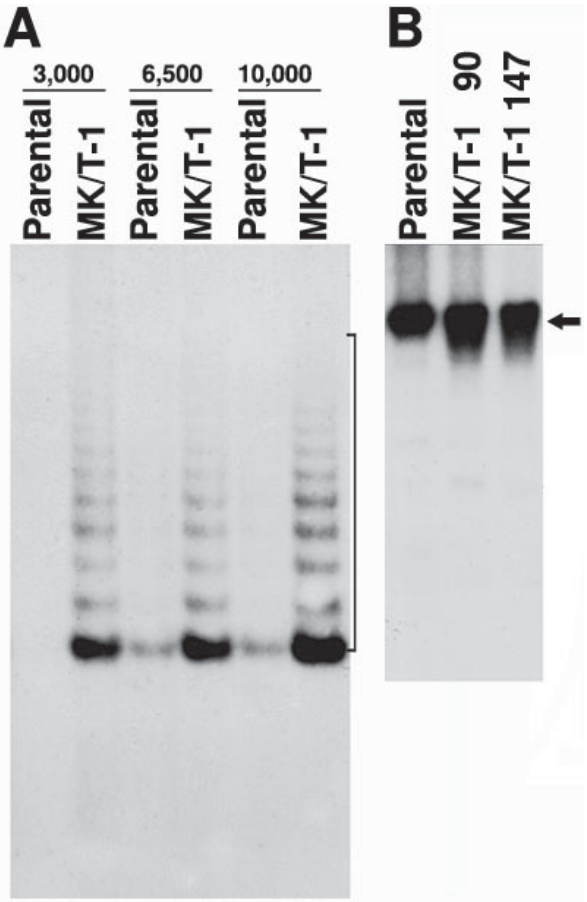

Figure 2. Telomerase analysis in MK/T-1 cells. **A**: Expression of telomerase activity in the MK/T-1 cells. TRAP assay of MK/T-1 cells (MK/T-1), showing the expression of telomerase activity appearing as the telomere elongation ladder pattern (indicated by bracket at right) compared to parental (Parental) untransfected corneal stroma derived cells at the same cell equivalents which exhibit only low baseline levels of telomerase activity. The cell equivalents used in each parallel TRAP assay reaction are indicated at the top. **B**: Assessment of relative telomere length (RTL) in parental versus MK/T-1 cells. Southern analysis was performed on *Sau3a I* digests of genomic DNA using a radiolabeled telomere repeat-specific oligonucleotide probe. The MK/T-1 cells at passages 90 and 147 (MK/T-1 passage 90 and 147) showed no difference in the electrophoretic mobility of the 23 kDa band hybridizing to the telomere repeat-specific oligonucleotide probe and representing RTL (arrowed) when compared to parental cell cultures shown in the first lane.

Lumican and keratocan probes used were as previously described, while full length coding sequence probes for mouse decorin and mimecan were generated by reverse transcriptase polymerase chain reaction (RT-PCR) using specific primers for mouse decorin and mimecan.

Western Blotting: Cell lysates were prepared using Triton-X 100 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton-X 100) supplemented with protease inhibitors (1 mM PMSF, 0.3 U/ml aprotinin, and 10 μg/ml leupeptin) and phosphatase inhibitors (1 mM sodium orthovanadate, 25 mM sodium fluoride, and 10 mM glycerophosphate, [30]). Lysates were clarified by centrifugation and protein was quantified and analyzed by SDS-PAGE. Gels were processed for western blotting using antibodies directed against vimentin, (Beohringer-Mannheim), α-tubulin (Sigma Immunochemicals), α-smooth muscle actin (Sigma Immunochemicals) and phospho-p38-MAPK (New England Biolabs, Beverly, MA). Incubations, washes and western blotting was performed by standard procedures using chemiluminescence detection (ECL Plus reagent, Amersham, Chicago, IL).

Immunocytochemistry: Immunocytochemical analysis was performed to assess the in situ patterns of regulation of α-smooth muscle actin expression in individual MK/T-1 cells after TGF-β2 treatment. Immunocytochemistry was performed with MK/T-1 cells plated onto glass coverslips using the same monoclonal antibody to ASMA (as used for western blotting above) and fluorescent (rhodamine) conjugated anti-mouse secondary antibody.

RESULTS

In an effort to derive an immortalized cell line using cell cultures prepared from corneal stroma, we have transfected a human telomerase reverse transcriptase subunit (hTERT)

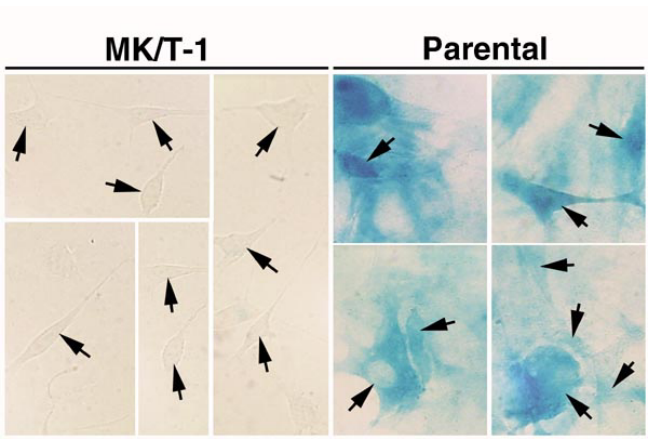

Figure 3. SA-β-Gal activity in MK/T-1 and parental cells. SA-β-Gal activity was not detected in passage 190 MK/T-1 cells (Left panels: MK/T-1) while the parental cells had already upregulated SA-β-Gal at high levels by passage 9 (Right panels: Parental). All preparations shown are representative fields and were incubated in identical SA-β-Gal staining solutions for equal lengths of time. Individual cells are arrowed in each panel.

cDNA expression construct to support immortalization of cells derived from mouse corneal stromal cultures in vitro. One subclone resulting from transfections of the mouse corneal stromal cell cultures with the hTERT vector was maintained through more than 100 consecutive passages in selective medium, was named MK/T-1 and further studied. The MK/T-1 cells have a morphology resembling the parental corneal stroma derived cells and display contact inhibition properties in culture. Phase contrast views Figure 1 of different passages of MK/T-1 cells growing in tissue culture dishes are compared to the parental untransfected cells at passage 5 which are nearing stages of senescence. As shown in Figure 1, late passage MK/T-1 cells are similar in appearance to early passage MK/T-1 cells and parental cells before senescence. The MK/T-1 cells are fibroblastic in morphology. No evidence of transformation foci was observed in the MK/T-1 cultures at any time, even despite prolonged maintenance under postconfluency conditions of separate samples of the cell line. Furthermore, the MK/T-1 cells failed to form colonies when cultured in soft agar, while IEM cells, an SV40 Large T transformed embryonic mouse endothelial cell line [25] formed high numbers of colonies. From platings of 2.5 x 10^5 and 1.25 x 10^5 IEM cells, 2798 and 1490 soft agar colonies, respectively, were counted while a starting number of 5 x 10^5 IEM cells formed a confluent lawn of colonies which could not be counted. In contrast, late passage MK/T-1 cells did not form any colonies after the same period of time at any of the three initial plating densities.

We next assessed telomerase activity, RTL and SAβ-Gal in the MK/T-1 cells and parental cells. As measured by the TRAP assay, the MK/T-1 cells express higher levels of telomerase activity when compared to parental cells which express only a low basal level of telomerase activity (Figure 2A). We also tested if late passages of the MK/T-1 cells displayed changes in RTL by Southern hybridization analysis using a radiolabeled telomere repeat specific oligonucleotide probe. The MK/T-1 cells at two different late passage numbers showed no discernable difference in RTL when compared to parental cell cultures (Figure 2B). Senescence associated β-galactosidase (SA-β-Gal) activity was not detected in the late passage MK/T-1 cells (passage 190) while the parental cells had already upregulated SABG at high levels by passage 9 (Figure 3).

MK/T-1 cells retain several gene expression characteristics consistent with a fibroblast cell phenotype. Western and Northern blotting was performed with early passage untransfected parental corneal stroma derived cells and with MK/T-1 cells at passages 50-90 using antibodies and probes for intermediate filaments, collagen I and proteoglycan markers known to be expressed by fibroblast cells. The MK/T-1 cells expressed vimentin, which is consistent with a fibroblast phenotype, and also expressed the cytoskeletal protein α-tubulin, which served as a positive control (Figure 4). The late passage MK/T-1 cells showed expression of mRNA for the lumican, mimecan, collagen I and decorin genes (Figure 5). The MK/T-1 cells did not express keratocan mRNA, as assessed by both Northern blotting and RT-PCR (data not shown).

Untreated MK/T-1 cells express very low levels of ASMA, a marker for fibrovascular and smooth muscle cells. The MK/

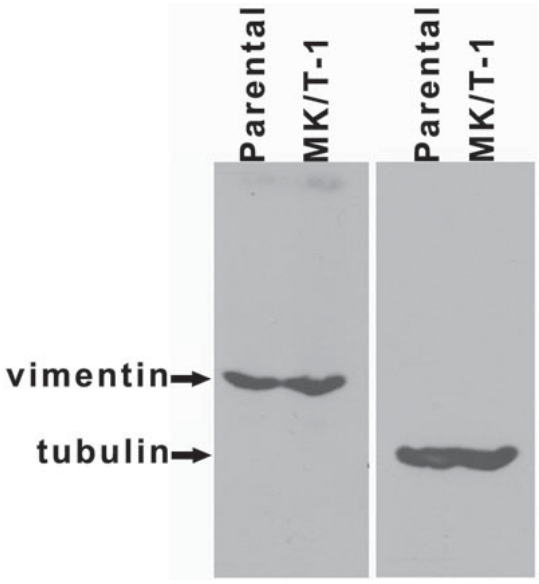

Figure 4. MK/T-1 cells display gene expression characteristics consistent with fibroblasts. Western blotting was performed with early passage untransfected parental cell lysates and lysates from MK/T-1 cells at passage 50 using antibodies to intermediate filament and cytoskeletal markers. The MK/T-1 cells expressed vimentin, which is consistent with a fibroblast phenotype. MK/T-1 expressed the ubiquitous cytoskeletal protein α-tubulin, which served as a positive control.

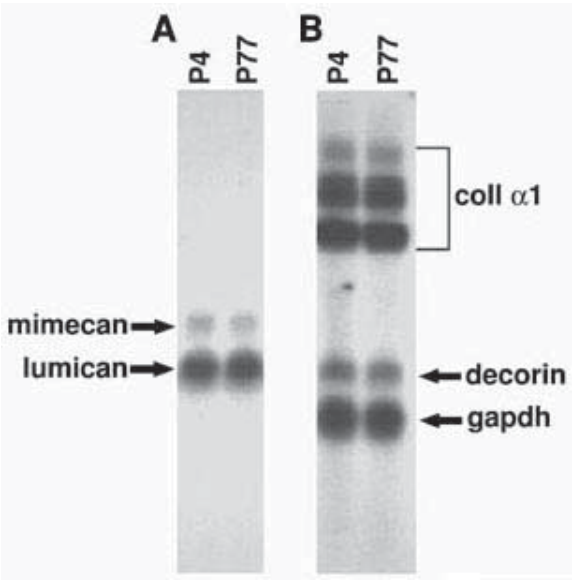

Figure 5. Proteoglycan and extracellular matrix gene expression in late passage MK/T-1 cells. Regulation of gene expression in early and late passage (P4 and P77, respectively) MK/T-1 cells was assessed by Northern blotting. The MK/T-1 cells expressed mRNA for lumican (**A**), mimecan (**A**), decorin (**B**), and collagen α1 (**B**). The two blots shown were produced with equal parts of the same RNA preparations. Glyceraldehyde-3-phosphate dehydrogenase (gapdh) was used as an internal control for loading and RNA integrity.

T-1 cells upregulated ASMA when cultured in the presence of TGF-β2 (Figure 6). Accompanying the TGF-β2 mediated induction of α-smooth muscle actin was the induction of activating phosphorylation of p38-MAPK (phospho-p38-MAPK) which is consistent with the activation of a signaling cascade stimulated by TGF-β2 (Figure 6). The induction of α-smooth muscle actin was detected both by western blot in cell lysates and was also observable by immunocytochemistry in situ. By immunocytochemistry, the induction of α-smooth muscle actin after treatment was detected in many of the cells cultured with TGF-β2 and was accompanied by spreading of these cells in which stress fibres positive for α-smooth muscle actin became visible (Figure 7A, see arrows), while untreated MK/T-1 cells expressed very low levels of α-smooth muscle actin after 24 h of culture (Figure 7B).

DISCUSSION

Overexpression of hTERT has been used previously to immortalize primary cell cultures without the added complication of oncogenenic transformation [31]. We reasoned that overexpression of telomerase activity might also aid in immortalizing cells derived using cultures of mouse corneal stroma and thus transfected a human telomerase reverse transcriptase subunit expression construct into cultured mouse corneal cells. A cell line was derived during these experiments and named MK/T-1. The MK/T-1 cells retain a morphology similar to the parental cells and display contact inhibition in culture. Since they failed to form colonies in soft agar, MK/T-1 cells do not display a transformed phenotype. Although the MK/T-1 cells express telomerase activity as assessed by TRAP analysis compared to cells from the parental cell cultures which express only low levels of hTERT, the late passage MK/T-1 cells did not show any differences in RTL when compared to parental cell cultures. Nevertheless, SA-β-Gal activity, a marker of cellular senescence, was not detected in the late passage MK/T-1 cells while the parental cells had already upregulated high levels of SA-β-Gal by passage 9. Since TERT is thought to suppress senescence and support cellular immortalization through maintenance of telomere length, our results suggest that events in addition to maintenance of hTERT expression may have contributed to the immortalization and inhibition of senescence of the MK/T-1 cell line. Our results do not exclude the possibility that hTERT alone is insufficient for immortalizing cells derived from mouse primary corneal stroma cultures. Therefore, other events such as spontaneous mutations in growth control genes may have contributed to immortalization of MK/T-1 cells. Nevertheless, our results indicate that cells initially derived from mouse corneal stroma cultures overexpressing hTERT retain a fibroblastic appearance in culture and do not appear to undergo cellular transformation in culture as assessed by anchorage independent growth. Since the MK/T-1 cells have undergone over 100 passages in vitro without signs of transformation, any events ex-

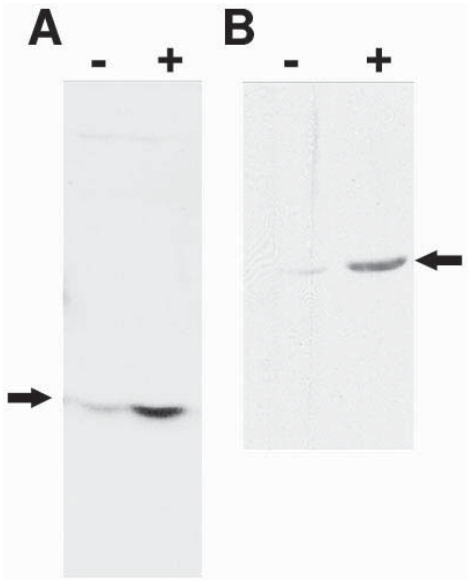

Figure 6. Regulation of gene expression in MK/T-1 cells by TGF-β2 treatment. Induction of activating phosphorylation of p38-MAPK (**A**), and induction of α-smooth muscle actin (SMA-α, **B**) by TGF-β. Western blot analysis with a rabbit anti-phospho-p38-MAPK antibody (**A**) and a mouse anti-SMA-α (**B**) of extracts (50 μg of protein) of MK/T-1 cells unstimulated (-) and stimulated (+) with TGF-β2. MK/T-1 cells treated in culture for 1 h with TGF-β2 showed a significant induction of phospho-p38-MAPK (plus lane in **A**), while the untreated MK/T-1 cells expressed very low levels of phospho-p38-MAPK at 1 h (minus lane in **A**). MK/T-1 cells treated in culture for 48 h with TGF-β2 showed a significant induction of SMA-a (plus lane in **B**), while the untreated MK/T-1 cells expressed very low levels of SMA-α at 48 h (minus lane in **B**). Arrows indicate positions of phospho-p38-MAPK and α-smooth muscle actin bands in **A** and **B**.

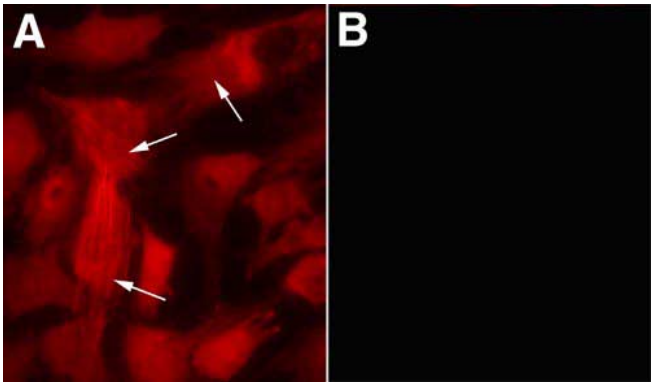

Figure 7. Immunocytochemical analysis of the induction of α-smooth muscle actin expression. Immunocytochemistry was performed with MK/T-1 cells using the same monoclonal antibody to α-smooth muscle actin (as used for western blotting in Figure 6) and fluorescent (rhodamine) conjugated anti-mouse second antibody. MK/T-1 cells expressed very low levels of α-smooth muscle actin after 24 h of culture (**B**), while MK/T-1 cultures treated for 24 h with TGF-β2 showed a significant increase in the numbers of cells expressing α-smooth muscle actin and the intensity of the actin signal (**A**). The TGF-β2 treated cultures also showed a change to a more spread out morphology consistent with reorganization of the cytoskeleton.

clusive to hTERT expression which support MK/T-1 immortalization do not appear to include oncogenic transformation.

The expression of vimentin, lumican, mimecan, collagen alpha 1, and decorin by the MK/T-1 cells is consistent with a fibroblast phenotype. That the MK/T-1 cells do not show keratocan expression is consistent with previous work of others demonstrating the conversion of keratocytes to fibroblasts and loss of keratocyte-like characteristics in culture [22]. However, the loss of keratocan expression also precludes the definitive classification of MK/T-1 cells as corneal, since fibroblasts from other tissue sources also do not express keratocan and display the same characteristics as MK/T-1 cells. Definitive classification of MK/T-1 cells as corneal must await further analysis and exploration of whether these cells might be capable of trans-differentiating into cells with keratocyte-like characteristics. These experiments are currently in progress in our laboratory. However, the MK/T-1 cells respond to cytokine stimulation in a manner consistent with previously reported responses of corneal fibroblasts. The untreated MK/T-1 cells expressed the ubiquitous cytoskeletal protein α-tubulin but either expressed very low levels or were negative for the expression of ASMA, which is a smooth muscle cell marker and marks cells which are transdifferentiating through a myofibroblastic phenotype. That the MK/T-1 cells upregulate ASMA and induce phosphorylation of activating p38-MAPK upon induction with TGF-β2 is consistent with activation of the TGF-β signal transduction response [32,33] and the shift in phenotype to myofibroblastic features. These cellular phenotypic changes are consistent with previously reported changes in morphology and α-smooth muscle actin induction in cultured corneal fibroblasts in vitro during TGF-β2 treatment [34-39]. Interestingly, similar changes are also observed during responses of corneal cells in situ during disease and injury [40-43]. Since fibroblasts are important cells in the processes of wound-healing and tissue remodeling, fibroblast cell lines such as MK/T-1 may provide isolated systems to help understand the molecular events associated with responses of fibroblast cells derived from specific tissues such as cornea.

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