Published in final edited form as:
FASEB J. 2005 August ; 19(10): 1371–1373. doi:10.1096/fj.04-2770fje.

PAX6 expression identifies progenitor cells for corneal keratocytes

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Abstract

Keratocytes of the corneal stroma produce a transparent extracellular matrix required for vision. During wound-healing and in vitro, keratocytes proliferate, becoming fibroblastic, and lose biosynthesis of unique corneal matrix components. This study sought identification of cells in the corneal stroma capable of assuming a keratocyte phenotype after extensive proliferation. About 3% of freshly isolated bovine stromal cells exhibited clonal growth. In low-mitogen media, selected clonal cultures displayed dendritic morphology and expressed high levels of keratan sulfate, aldehyde dehydrogenase 3A1, and keratocan, molecular markers of keratocyte phenotype. In protein-free media, both primary keratocytes and selected clonal cells aggregated to form attachment-independent spheroids expressing elevated levels of those marker molecules. The selected clonal cells exhibited normal karyotype and underwent replicative senescence after 65–70 population doublings; however, they continued expression of keratocyte phenotypic markers throughout their replicative life span. The progenitor cells expressed elevated mRNA for several genes characteristic of stem cells and also for genes expressed during ocular development PAX6, Six2, and Six3. PAX6 protein was detected in the cultured progenitor cells and a small number of stromal cells in intact tissue but was absent in cultured keratocytes and fibroblasts. Cytometry demonstrated PAX6 protein in 4% of freshly isolated stromal cells. These results demonstrate the presence of a previously unrecognized population of PAX6-positive cells in adult corneal stroma that maintain the potential to assume a keratocyte phenotype even after extensive replication. The presence of such progenitor cells has implications for corneal biology and for cell-based therapies targeting corneal scarring.

Keywords

cornea; keratan sulfate; stem cell

Transparency of the cornea is conferred by the highly organized extracellular matrix of the corneal stroma. Parallel bundles of collagen fibers of highly uniform diameter are tightly packed in lamellae lying parallel to the corneal surface. A group of cornea-specific proteoglycans associate with stromal collagen effecting a supramolecular structure permitting transmission of light (1–3). Keratocytes, quiescent cells of neural crest origin, lie sandwiched between the stromal lamellae, extending dendritic processes to form an interconnected cellular network throughout the tissue (4). Keratocytes are responsible for deposition of the stromal extracellular matrix during late embryonic development, and these cells continue to maintain the integrity of the transparent matrix in the adult.

In response to wounds, infections, and various chronic corneal pathologies, keratocytes become motile, mitotically active, and adopt a fibroblastic phenotype in which they secrete fibrotic

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matrix components that disrupt corneal transparency (5,6). Such corneal scars are long-lasting and represent a visual impairment for millions of individuals worldwide (7). Keratocyte biology, particularly the understanding of environmental influences on the expression of matrix, has consequently been a focus of numerous studies over several decades.

Early studies of keratocytes in vitro described these cells as growing with a typical fibroblastic morphology and rapidly ceasing the secretion of keratan sulfate, a glycosaminoglycan present in unique abundance in the stroma (8,9). More recently, quiescent primary keratocytes cultured in serum-free medium or media with low-mitogen horse serum were observed to maintain dendritic morphology and secretion of keratan sulfate as well as the corneal-specific proteoglycan keratocan (10). In culture media containing fetal bovine serum (FBS), primary keratocytes rapidly lose their characteristic phenotype, but in reduced serum supplemented with fibroblast growth factor-2 (FGF), keratocytes proliferate and maintain expression of their phenotypic markers (11). Serial passage in reduced serum-FGF medium, nevertheless, led to a slower, but inexorable, loss of the keratocyte phenotype (11).

In the past decade, several studies have documented, in a variety of adult tissues, the presence of cells maintaining an ability to divide and give rise to fully differentiated daughter cell populations (12–22). In some tissues, these cells exhibit pluripotent potential for differentiation into any of several different cell types and as such constitute true adult stem cells (23–25). In other tissues, the precursor cells maintain an extensive proliferative potential but exhibit a restricted cell fate. Such proliferative precursor cells are thought to provide a cellular reservoir for tissue renewal.

In the current study, we sought to determine if a similar population of cells representing keratocyte progenitors could be identified in adult bovine corneal stroma. We report the presence in the stroma of a population of cells capable of clonal growth, expressing genes characteristic of adult stem cells and also genes present in embryonic ocular tissue. These cells do not lose the ability to express molecular markers of the keratocyte phenotype even after extensive proliferation in vitro.

METHODS AND MATERIALS

Cell culture

Primary stromal cells were obtained from fresh bovine corneas by collagenase digestion as described previously (26). The cells were diluted in serum-free DME/F-12 medium containing antibiotics and cultured on tissue culture-treated plastic at 4×10^4 cells/cm^2 in a humidified atmosphere containing 5% CO_2 (26). Culture medium was changed after 24 h (day 1) to fresh DMEM/F-12 with antibiotics (26). Heparin-stripped horse serum was prepared from 50 ml platelet-poor horse serum (Sigma-Aldrich, P5552) by passage at room temperature over a 10 ml column of heparin-Sepharose (Sigma-Aldrich, H0402). The resulting serum had little mitogenic activity on keratocytes. Fibroblasts were obtained by plating primary keratocytes at 1×10^4 cell/cm^2 in DME/F-12 containing 10% fetal bovine serum. Cells were passaged serially 1:3 for four to seven times. Confluent clonal cell and fibroblast cultures were incubated for 1 wk in 1% low-mitogen horse serum in DME/F12 with antibiotics (differentiation medium) to induce keratocyte differentiation.

Cloning of stromal cells was carried out on freshly isolated primary cells or uncultured primary cells previously frozen in liquid nitrogen. Cells were cloned by limiting dilution in which an average of 0.3 cells per well were plated in cloning medium (CM) consisting of DME/F-12 with antibiotics, 0.1 mM ascorbate-2-phosphate, 2% FBS, 10 ng/ml epidermal growth factor (EGF), 10 ng/ml platelet derived growth factor BB (PDGF), 10 ng/ml fibroblast growth factor-2 (FGF), and 5 ug/ml insulin, 5 ug/ml transferrin, and 5 ng/ml selenous acid (ITS, Invitrogen).

Cell growth after 3 wk was assessed using a fluorometric Alamar Blue assay (Accurate Chemical and Scientific, Westbury, NY) according to the manufacturer's procedure. Cultures were expanded as the wells became confluent.

Histology and cytology

Morphology of living cells was observed by phase contrast microscopy using a ×10 objective. For actin filament analysis, cells were fixed in room temperature 3.2% paraformaldehyde and double-stained with Alexa 488 phalloidin (Molecular Probes) and anti-vinculin clone hVIN-1 (Sigma-Aldrich) followed by goat anti-mouse labeled with Alexa 546 (Molecular Probes) using procedures described previously (26). PAX6 was observed in cultured, fixed and permeabilized cells doubly stained with polyclonal anti-PAX6 (Covance, Berkeley, CA) and mouse anti-cytoplasmic myosin CMII25 (27; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) followed by Alexa 488 anti-rabbit and Alexa 543 anti-mouse secondary (Molecular Probes). For determination of PAX6 expression in the intact stroma, whole bovine cornea was fixed as above and cut into quarters. Stroma was separated into anterior and posterior portions mechanically, and PAX6 was stained as described above. Cell membranes of keratocytes were counterstained using the red-fluorescent carbocyanine lipophilic tracer DiD at 50 μM (Molecular Probes). Live and dead cells in spheroids were stained using calcein AM (Molecular Probes) at 50 μM for 30 min at 37°C in serum-free culture medium followed by 5 min with 5 μg/ml propidium iodide. Keratocan was stained using a peptide antibody to keratocan (28) provided by Dr. Chia-Yang Liu. Photographs were acquired on a Bio-Rad Radiance Plus Laser Scanning Confocal microscope using oil objectives.

Staining for flow cytometry was carried out on 200,000 primary stromal cells fixed in Cytofix/Cytoperm (BD Biosciences) and blocked in 5% heat-treated normal goat serum in BD Perm/Wash buffer. Cells were stained with PAX6 antibody or nonimmune rabbit IgG control as primaries followed by secondary goat-anti-rabbit Alexa 488. The stained, fixed cells were processed on a BDL SRII Flow Cytometer, and the data were analyzed using WinMDI software.

PCR

Quantitative RT-PCR for keratocan and aldehyde dehydrogenase 3A1 (ALDH) was carried out as described previously (29). Briefly, RNA was isolated from cells using an RNeasy Mini kit (Qiagen) and cDNA was transcribed using random hexamers with SuperScript II reverse transcriptase (Invitrogen). PCR was carried out for 40 cycles of 15 s at 95°, 60 s at 60° after an initial incubation at 95° for 10 min in an ABI 7700 TaqMan thermocycler, using primers and FAM/TAMRA labeled hybridization probes for keratocan and ALDH (29). Gene-specific Ct values were standardized based on ribosomal 18S Ct values obtained for each cDNA. Values shown represent average and standard deviation of triplicate analyses from single RNA samples. Quantitative analysis of stem-cell genes was carried out using similar reactions with detection of amplified product by SYBR green dye. After amplification, melting curves were carried out on each sample to confirm homogeneity of the amplified DNA. Gel electrophoresis confirmed the amplified products to be of the expected size. Primers used in these assays are given in Table 1.

Immunoblot analysis

Proteoglycans in the culture medium were purified by ion exchange chromatography, dialyzed against water, and lyophilized (26). Samples normalized for cell number (by total cell DNA content) were transferred directly to PVDF membrane (dot blotting) or separated on 4–20% SDS-PAGE gels followed by electro-transferal to PVDF membranes. The membranes were then subjected to immunodetection using monoclonal antibody J19 against keratan sulfate (30) or polyclonal antibody to keratocan peptide (28). Confirmation of keratan sulfate on the

blots was obtained by treatment of samples before electrophoresis with mixed endo-β-galactosidase and keratanase II as described previously (29).

Cytogenetic karyotyping was carried out on metaphase spreads produced using standard techniques (31) from three different progenitor cell lines. Chromosomes were stained using propidium iodide (as above), and 25–30 chromosomal spreads were photographed and analyzed for chromosome number and size distribution for each cell line.

RESULTS

Keratocyte progenitor cells

To identify keratocyte progenitor cells we initially screened for cells capable of clonal growth, which maintained characteristic morphology and extracellular matrix secretion typical of keratocytes. With the use of a culture medium reported to support expansion of mesenchymal stem cells (32), typically 3% of freshly isolated bovine stromal cells were found to grow when plated at limiting dilution. At confluence in 96-well culture plates, the cloned cells had undergone ~14 cumulative population doublings (CPD) and little keratan sulfate was detected. The cloned cells exhibited a variety of cellular morphologies ranging from spindle shaped (fibroblastic) illustrated in Fig. 1A to the dendritic character, Fig. 1B, typical of differentiated keratocytes. Abundance of proteoglycans modified with keratan sulfate chains is a defining feature of the corneal stroma, and secretion of these molecules by corneal cells has not been previously demonstrated in culture beyond a few population doublings (10,11,33). We observed that shifting the cloned cells to a low-mitogen medium resulted in expression of keratan sulfate-containing proteoglycans in about one-half of the cultures (Fig. 1C). Although there was not a strict correspondence between dendritic morphology and keratan sulfate synthesis, a number of clones exhibited both and several of these were expanded for further analysis.

Confirmation of secretion of high molecular weight keratan sulfate proteoglycan by three selected clones is shown in Fig. 2A. This reactivity was eliminated by digestion with keratanase and endo-β-galactosidase, confirming these bands as keratan sulfate proteoglycans. In the cloned cells, cellular pool levels of mRNA for the proteoglycan protein keratocan and for the corneal crystallin ALDH were similar to those measured in primary keratocyte cultures and 50- to 200- fold greater than passage-four fibroblasts derived from primary keratocytes (Fig. 2B). Quiescent keratocytes exhibit a characteristic dendritic morphology, with filamentous actin associated primarily with the dendritic processes and little or no formation of focal adhesions (Fig. 2C). High passaged clonal stromal cells continued to exhibit a similar morphology (Fig. 2D). Mass cultures of keratocytes, however, passaged four times in 10% FBS-containing medium displayed a markedly different cytoskeletal morphology (Fig. 2E). In these fibroblastic cells, prominent actin cables cross the center of the cells and were anchored at focal adhesions containing vinculin.

In the course of this study, we observed that both primary keratocytes and the clonal keratocyte progenitors, when cultured as monolayers in FGF under serum-free conditions (Fig. 3A), begin to aggregate (Fig. 3B) forming rounded spheroids (Fig. 3C) that eventually float free of the substratum (Fig. 3D). Staining of the live spheroids with the vital dye calcein AM showed that they consist of solid balls of viable cells (Fig. 3E, green). Dead cells were observed typically only at the periphery of the spheroids (Fig. 3E, red). Immunostaining of the spheroids found keratocan protein throughout the assembly (Fig. 3F, red), whereas keratan sulfate was exclusively extracellular and at the periphery (Fig. 3F, green). Figure 3 shows spheroids formed by primary keratocytes. Similar or identical results were observed using spheres formed from clonal cells (not shown). Analysis of mRNA levels of the spheroids showed (Fig. 3G) these aggregates to express about twice the amount of keratocan mRNA as nonaggregated adherent

cells. Although levels were less than those in freshly isolated keratocytes, they were at least 20-fold greater than those of fibroblasts.

The three clonal lines chosen for assay all exhibited a normal karyotype of 60 chromosomes (data not shown), and, when passaged serially, all reached replicative senescence at 65–70 CPD (Fig. 4). Primary keratocytes in cloning medium expressed relatively low levels of ALDH and keratocan as did progenitor cells at both high and low passages (Fig. 5, open bars). When the cells were transferred to low-mitogen differentiation medium, expression levels of these two genes were increased in all three cultures (Fig. 5, shaded bars). The high passage progenitor cells (CPD60) were not as strongly up-regulated as the younger cultures (CPD27), however, suggesting a reduction in the differentiation response as the cultures age. Even in the CPD60 cultures, the mRNA levels were 10-fold greater than that of fibroblasts in similar media (not shown).

Stem cell gene expression

A number of studies have identified genes up-regulated in stem and progenitor cells of various adult tissues. Using RT-PCR, we screened the clonal progenitor cells for expression of 26 such genes and, of these, identified 10 genes expressed in each of these lines. Assessment of the mRNA levels for these genes in progenitor cells was compared with primary cultured keratocytes. As shown in Fig. 6, progenitor cell mRNA was enriched in genes known to be associated with stem cells such as Bmi1, CD90 (Thy1), CD73, CD166, ABCG2, Fhl1, stem cell factor (SCF, kit ligand), and Notch 1. Up-regulation of genes involved in early neural crest and ocular development, Six2, Six3, and PAX6 was also observed. The mRNA for the housekeeping gene GAPDH was only moderately altered in the progenitor cells, and, as shown above, keratocan and ALDH, markers of keratocyte differentiation, were reduced in these cells compared with keratocytes.

PAX6 is a transcription factor essential to ocular development and is expressed in several embryonic ocular tissues but not in adult keratocytes (34). Progenitor cultures showed strong nuclear immunostaining for PAX6 protein in the majority of the cells (Fig. 7A); however, primary keratocytes cultured under the same conditions had little PAX6 protein (Fig. 7B). Staining of bovine cornea showed nuclear localization of PAX6 only in rare individual stromal cells distributed throughout the stroma (Fig. 7C). Analysis of freshly isolated stromal cells by flow cytometry showed ~4% of the total to stain with the same antibody to PAX6 (Fig. 7D).

DISCUSSION

In this study, we report the presence of cells in the corneal stroma capable of clonal growth and extensive expansion in vitro while maintaining the ability to adopt both morphology and phenotypic marker expression unique to corneal keratocytes. This behavior clearly distinguishes these cells from keratocytes that exhibit a rapid loss of phenotype in response to expansion in vitro. The progenitor cells were selected based on their ability to grow clonally, display a dendritic morphology, and express keratan sulfate when shifted to low-mitogen media (Fig. 1). Further analysis confirmed the induction of a keratocyte-like phenotype in low-mitogen media with expression of mRNA for keratocan and ALDH at levels similar to that of primary keratocytes in culture and exhibition of a cytoskeletal morphology similar to that of primary keratocytes.

Progenitor cells and primary keratocytes also share a cellular behavior not previously reported. Monolayer cultures of both cell types spontaneously aggregate, forming attachment-independent spheroids. These spheroids are solid balls of viable cells expressing high levels of keratocyte-specific genes. Spheroid formation is common in transformed and tumorogenic cells but is not typical of attachment-dependent mesenchymal cells (35). Keratocyte-derived

fibroblasts did not form spheroids under the same conditions. Full understanding of implications of this cellular assembly will require more detailed investigation; however, for the purposes of this study, the formation of spheroids represents a specialized property of keratocytes that is lost after growth in vitro but maintained by the cloned progenitor cells. This property, along with expression of keratocyte extracellular matrix and assumption of the classic keratocyte dendritic morphology, shows that the cloned cells can adopt a phenotype indistinguishable from that of primary keratocytes. We found that maintenance of keratocyte phenotype could not be replicated in mass cultures of keratocytes simply by manipulation of culture conditions (11) nor was it shared by all clonal cultures (Fig. 1C). We therefore believe to have identified a discrete population of cells comprising a small fraction of the total cells of the corneal stoma.

Distinction between the progenitor cells and keratocytes was furthered by discovery of several stem cell-associated genes, differentially expressed in these cells. The cells were screened using RT-PCR for expression of a number of genes known to be up-regulated in multiple stem cell types. Of these, transcripts of eight genes were found to be up-regulated in the keratocyte precursor cells compared with keratocytes. Notch1 and Bmi1 have been linked to maintenance and self-renewal of stem cell populations in both mesenchymal and neural stem cells (36–41). SCF is a growth factor required for maintenance of hematopoietic stem cells and is also produced by mesenchymal stem cells (42). Four and a half LIM (FHL1) is a transcription factor identified in microarray studies as up-regulated in embryonic, neural, hematopoietic, and dermal stem cells (43,44). ABCG2 is a one of a family of multidrug transporter proteins present in several types of stem cells and cancer cells involved in xenobiotic removal. ABCG2 is responsible for Hoechst dye clearance by stem cells, a function used in isolation of “side population” cells by cell sorting (40,45,46). CD73, CD90, and CD166 similarly have been used in cell sorting-based selection of hematopoietic and mesenchymal stem cells (47–51). Up-regulation of these genes in the clonal cells strengthens the hypothesis that these cells represent a stem cell-like population in the corneal stroma phenotypically distinct from keratocytes.

In addition to stem-cell related genes, we observed strong up-regulation of three genes associated with eye development. PAX6, Six3, and Six2 are orthologs of drosophila homeobox genes essential for eye development. All three of these genes along with BMI1 and Notch1 are expressed in the neural plate of early vertebrate embryos. After migration of neural crest cells, Six2 transcripts are found in a variety of non-neuronal connective tissues, including the eye, beginning at mouse embryonic day 8 and continuing until about day 16, after which Six2 expression vanishes (52). In the same developmental stages, Six3 expression is limited to the retina, lens, hypothalamus, and pituitary (53). Six3 expression diminishes or is absent in most adult ocular tissues except for the retina. PAX6 is the best characterized of these factors in terms of ocular function. PAX6 expression is required for lens placode formation during early eye development, and haploinsufficiency of PAX6 expression results in small eyes or aniridia (54). PAX6 is expressed in retina and corneal epithelium throughout development. Unlike the Six genes, PAX6 expression continues in adult corneal epithelium. Studies on expression of these genes in developing vertebrates are consistent with the conclusion that none are expressed in the corneal stroma either during development or in adult tissue. Our RT-PCR analysis confirms previous findings showing the absence of expression of these genes in adult keratocytes. Immunostaining found PAX6 to be not detectable in keratocytes, consistent with the low mRNA levels of this gene. The majority of the cultured progenitor cells, on the other hand, showed high levels of nuclear PAX6 protein. In the corneal stroma, rare cells were observed to express PAX6 (Fig. 7C). The presence of a discrete population of PAX6 expressing cells in the stroma was confirmed by flow cytometry. We believe that this PAX6 positive population of cells in the stroma is the source of the progenitor cells we have isolated and characterized in vitro.

The high level of expression of embryonic genes in the clonal stromal cells reinforces the idea that these cells are representatives of a population of embryonic precursor cells clearly distinct from adult keratocytes. Because these genes mark cells involved in ocular differentiation, their expression in the clonal cells suggests that these cells may not be pluripotent stem cells but rather a population of cells committed to a specific differentiation lineage. The replicative life span of the clonal cells is consistent with this idea. Some pluripotent stem cell populations exhibit a very high replicative life span of 200–300 CPD. The onset of replicative senescence after 65–70 CPD in the corneal precursors is more representative of that of normal somatic bovine cells in culture (55). This finding is consistent with the hypothesis that the cells are not stem cells but stromal progenitor cells, with a committed cell fate and limited replicative life.

The biological role of progenitor cells in the corneal stroma is yet to be determined. In response to acute injury, cells near the wound become active, divide, and migrate into the injury area. The migrating cells produce inappropriate matrix components and reduced corneal ALDH leading to reduced corneal transparency. Because of the large number of cells involved in response to acute trauma, it seems likely that keratocytes are the primary responders. This idea is consistent with the finding that the phenotype of cells in healing wounds is similar to that of keratocyte-derived fibroblasts. There is indirect evidence for a cellular process involved in replacement and renewal of keratocytes different from that of acute wound healing. Mouse corneal epithelial scrape wounds repopulate with keratocytes within several days; however, 3 months is required before stromal cells regain keratocan expression (56). Acellular epikeratoplasty grafts in humans repopulate with apparently normal keratocytes without scar formation over a time period of 4 years (57). Similarly, opacities in corneal grafts in patients with familial diseases such as keratoconus, mucopolysaccharidoses, and corneal dystrophies can recur after periods ranging from 5–20 years (58–60). All of these results suggest a continuous slow process of replacement of keratocytes in the stroma. Although the existence of such a process has yet to be unequivocally demonstrated, progenitor cells in the stroma possessing a high replicative potential could provide the reservoir for this kind of renewal and replacement of keratocytes.

In summary, this study has identified a population of cells present in adult mammalian corneal stroma with the ability to divide extensively, generating differentiated keratocytes. Both in vivo and in culture these progenitor cells express the PAX6 gene, a marker of ocular progenitor cells not expressed by differentiated keratocytes. Understanding the properties of these cells will aid understanding of corneal wound healing and repair processes. Expansion of these cells in vitro may provide a source of cells useful in cell-based corneal therapy or for development of bioengineered corneas.

Acknowledgments

The authors wish to thank Carolyn J. Anthon for excellent technical assistance with karyotyping and Dr. Chai-Yang Liu for the donation of antibody to keratocan. This work was supported by National Institutes of Health Grant EY09368, EY013806 (to J. L. Funderburgh), EY003263 (to N. SundarRaj), 30-EY08098 (University of Pittsburgh, Department of Ophthalmology Core Grant), Research to Prevent Blindness, and the Eye and Ear Foundation of Pittsburgh. J. L. Funderburgh is a Jules and Doris Stein Research to Prevent Blindness Professor.

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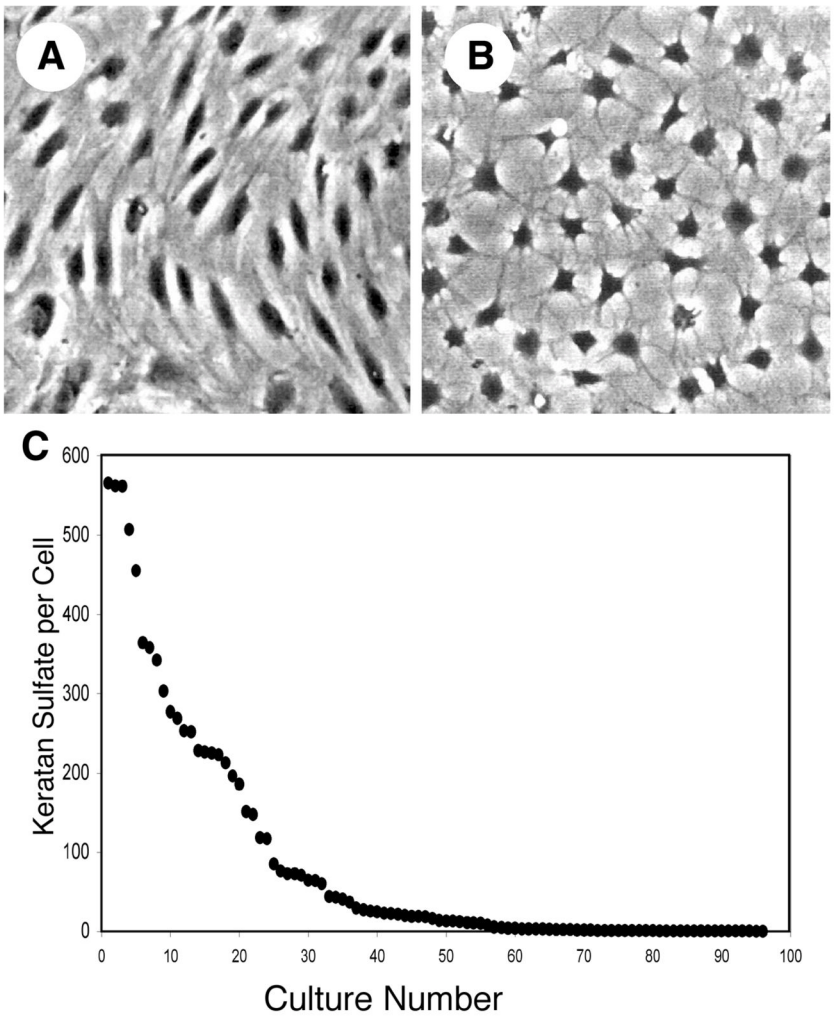

Figure 1.

Properties of clonal cultures from corneal stroma. Phase contrast micrographs illustrate the range of cellular morphologies observed in cells growing clonally from bovine corneal stroma, fibroblastic **(*A*)** and dendritic **(*B*)**. ***C*)** Keratan sulfate was detected by dot blot in proteoglycans isolated by 96 different clones 2 wk after a shift to low-mitogen media. The amount of keratan sulfate is calculated relative to cell abundance.

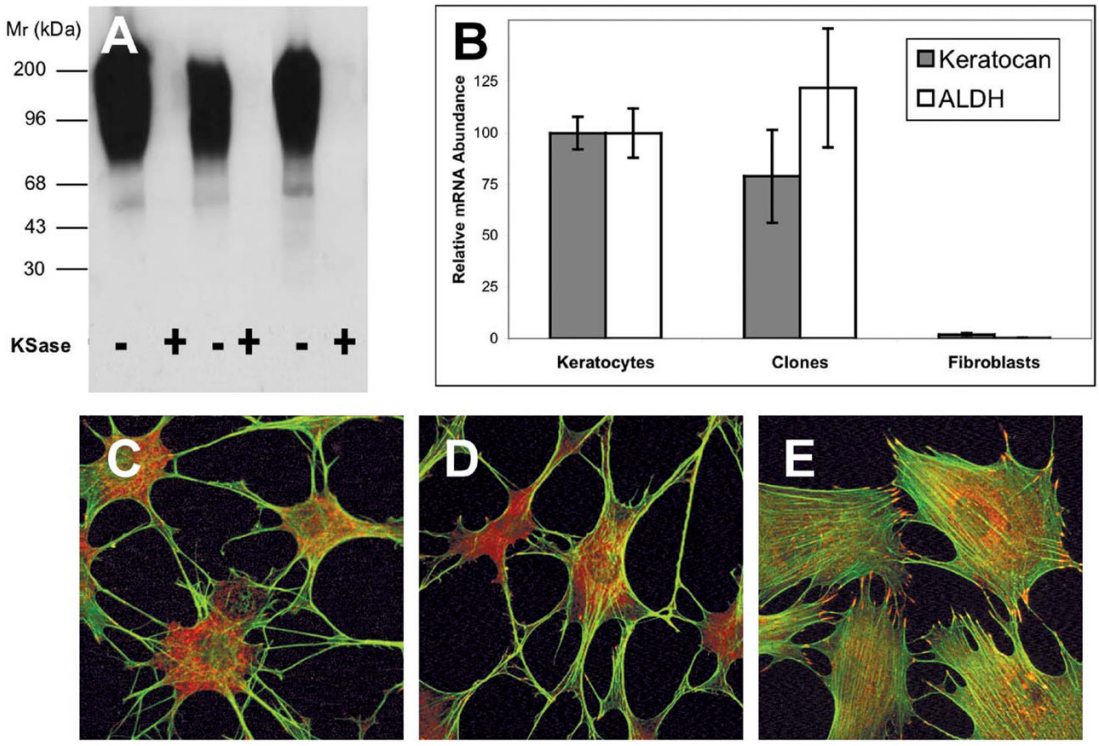

Figure 2. Phenotype of cloned stromal cells

A) Proteoglycans from culture media of 3 selected stromal clones (CPD 22) were assayed for keratan sulfate-containing proteoglycan using monoclonal antibody J19 with (+) or without (−) pretreatment with keratanase. ***B)*** mRNA pools for keratocan (shaded) and ALDH (open) were determined by quantitative RT-PCR in primary keratocytes, clonal stromal cells (CPD 24), and passage-four fibroblasts. Values are normalized to keratocytes = 100. ***C)*** Primary keratocytes were immunostained for f-actin (green) and vinculin (red). ***D)*** Clonal stromal cells (CDP 50) were stained similarly to ***C. E)*** Fibroblasts, passage four, were stained similarly to ***C*** and ***D***.

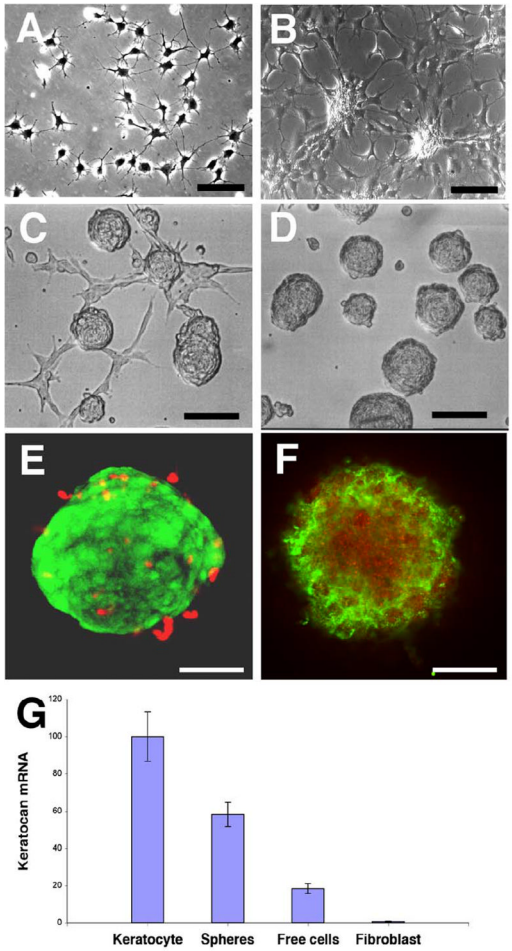

Figure 3.

Spheroid formation by stromal cells. Primary keratocytes in mitogen-free medium **(*A*)** attach and spread with a dendritic morphology. After 1 wk in medium containing 10 ng/ml FGF and ITS (***B*),** cells form refractile aggregates. After 3 wk (***C***), aggregates become spherical and could be readily separated from individual cells (***D***). Live cells in spheres were stained with calcein AM (green) and propidium iodide (red) to show live and dead cells (***E***). Fixed spheres (***F***) were stained for keratan sulfate (green) and keratocan (red). Photos ***E*** and ***F*** are optical sections through the center of a sphere using confocal microscopy as described in Materials and Methods. RNA from spheres and from cells not aggregated into spheres from the same culture was subjected to analysis by quantitative RT-PCR for keratocan mRNA as described in Materials and Methods. mRNA pools were compared with those of noncultured stromal cells and with corneal fibroblasts in ***G***. Bars in ***A–D*** = 100 μm and in ***E–F*** = 50 μm.

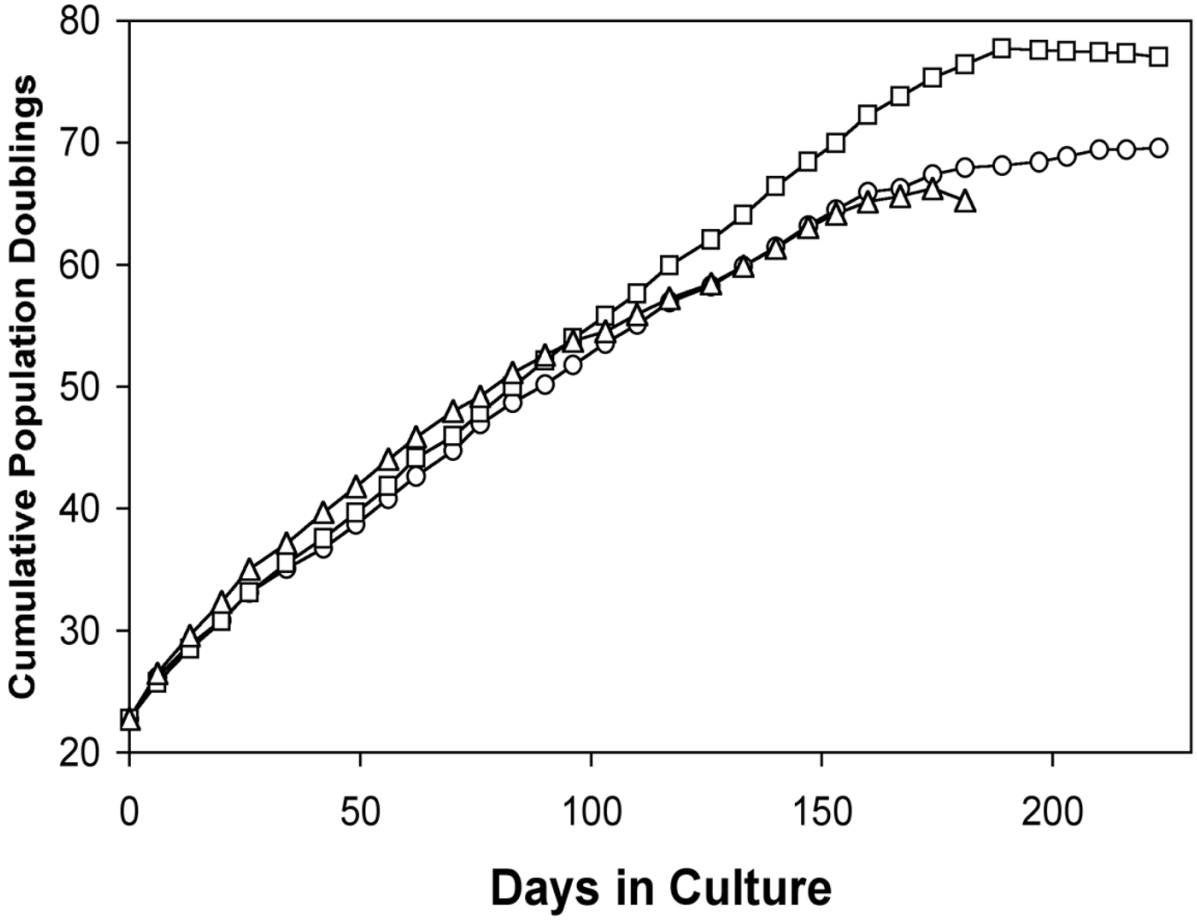

Figure 4.

Replicative lifespan of cloned progenitor cells. After the initial 6 passages, estimated to require 22 CPD, 3 clonal progenitor lines were passaged at 7 day intervals by trypsinizing, counting, and replating at a fixed density of 1×10^4 cells/cm^2. Points represent population doublings calculated from average cell counts of 2 cultures from each cell line.

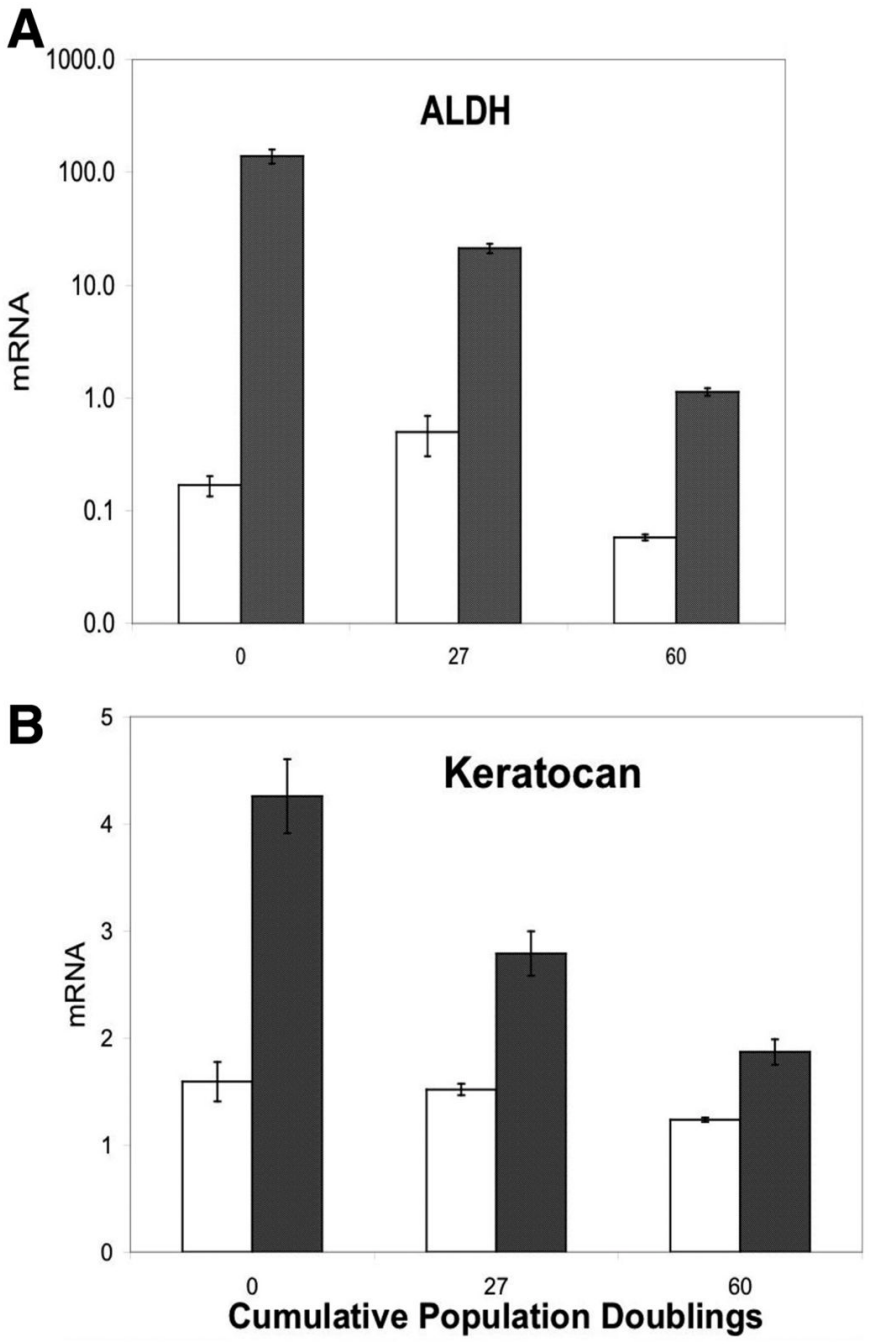

Figure 5.

Marker expression during expansion of progenitor cells. RNA was purified from primary keratocytes (CPD 0) and from clonal progenitor cells at early passage (27 CPD) and late passage (60 CPD). Cells were maintained in cloning medium (CM) (open bars) or after a week in differentiation medium (solid bars). Relative mRNA abundance was calculated as a percentage of that of primary keratocytes. ***A)*** ALDH mRNA. ***B)*** keratocan mRNA.

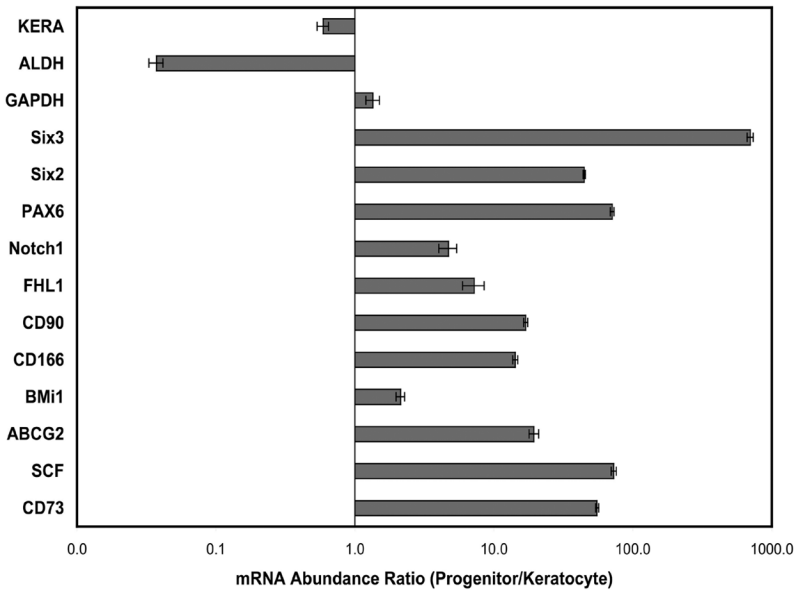

Figure 6.

Stem cell gene expression by stromal progenitor cells. Relative abundance of mRNA for 11 genes reported in stem and ocular progenitor cells was examined using quantitative RT-PCR in clonal stromal (progenitor) cells compared with primary keratocytes as described in Materials and Methods. As controls, GAPDH, keratocan (KERA), and ALDH were determined in the same samples.

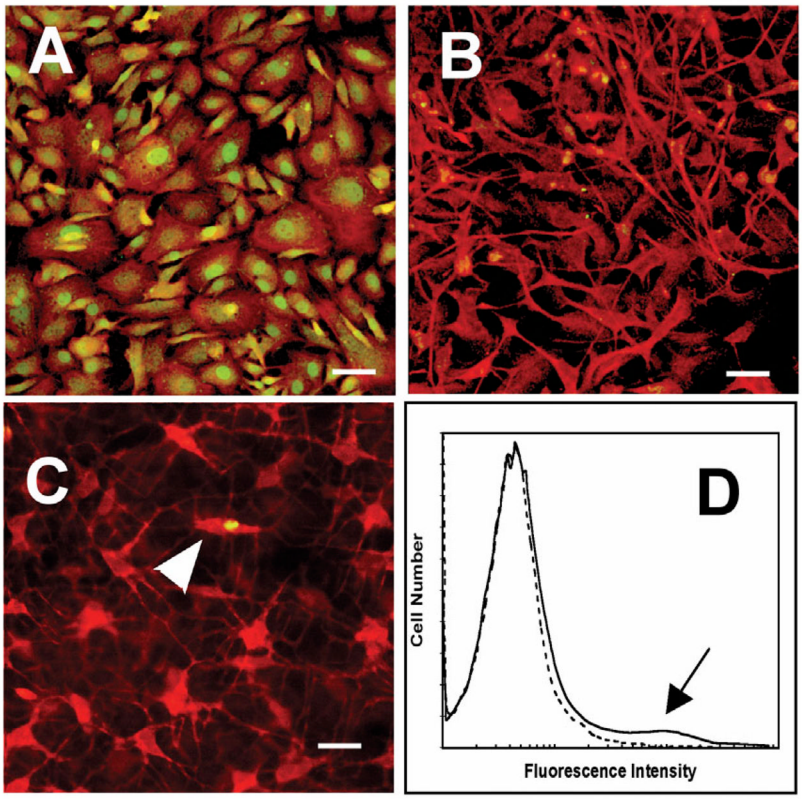

Figure 7.

PAX6 expression by stromal progenitor cells. ***A)*** PAX6 protein in cloned progenitor cells was immunostained (green) and counterstained for cytoplasmic myosin (red). ***B)*** PAX6 protein was not detected in primary keratocytes cultured under identical conditions. ***C)*** PAX6 was detected only in rare cells in intact corneal stroma (arrowhead). Keratocyte cell membranes were counterstained with DiD (red). ***D)*** Freshly isolated stromal cells were fixed and stained for PAX6 protein and then analyzed by flow cytometry. Solid line shows results with a PAX6 primary antibody. Dashed line shows a nonimmune rabbit IgG primary. Fluorescence intensity is shown as a logarithmic scale. Arrow indicates a population of stained cells representing 4% of the total. White bars = 50 μm.

Table 1

Primers for stem cell gene RT-PCR

Gene	Source*	Forward	Reverse
ABCG2	NM_174222	AACTTCTGCCCAGGACTCAA	ATGCCAAGGCTACGTGATTC
CD166	NM_174238	TCTCATGTTTGGCAAATGGA	GAAATGGGGCTTTGCTTACA
CD73	NM_174129	CTGAGACACCCGGATGAGAT	ACTGGACCAGGTCAAAGGTG
CD90	TC288067	ATACCCCTCCATCCTTCCAC	CACCTCTGCCAATACCACCT
Fhl1	TC262429	TGGCACAAAGACTGCTTCAC	TCTTGCATCCAGCACACTTC
SCF	AB033716	GGGATCTGCAGTAACCGTGT	TTCCACCATCTCGCTTATCC
Six2	TC297269	CTCTCTTCCTTTGCCCTCCT	CCGAGAAACACTGAGGGGTA
Six3	BF654243	ACCATCAACAACTCCCAACC	AGCGGTGCTTGTCCTAGAAA
Bmi1/Flvi-2	TC279460	CTCCACCTCTTCCTGTTTGC	CCAGATGAAGTTGCTGACGA
Notch1	TC263497	GATGTGGTTCATGCATTTCG	CACACCTCCGACTTCTCACA
PAX6	U73621	TTTTCCTCCCTCCCTAACTCA	GATGCGTCAAAACGAAGTCA

* Source numbers starting with “TC” were obtained from The Institute for Genome Research Cattle Gene Index (www.tigr.org). Others are GenBank accession numbers.