Preservation of the Limbal Stem Cell Phenotype by Appropriate Culture Techniques

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PURPOSE. To evaluate the effect of several culture variables on clonal growth and differentiation of limbal stem cells ex vivo and provide an improved culture technique that supports preferential expansion and preservation of stem cells for therapeutic applications.

METHODS. Corneal epithelial stem cells were isolated from human limbal specimens and clonally expanded on a 3T3 feeder layer, followed by subcultivation of holoclones on fibrin gels. The effect of different limbal regions, enzymatic dissociation methods, and culture media supplemented with different calcium, serum, and growth factor concentrations on colony-forming efficiency, colony size, and colony density was compared. A panel of putative stem cell and differentiation markers was used to analyze the epithelial phenotype by morphologic and immunohistochemical methods.

RESULTS. Limbal cells obtained from the superior limbus, isolated by a two-step enzymatic dissociation method (dispase II/trypsin-EDTA), and cultured in low to medium (0.03–0.4 mM) calcium concentrations with proper serum levels (10% FCS) and growth factor combinations (EGF, NGF) yielded the highest clonal growth capacity and an undifferentiated cellular phenotype. Subcultivation of holoclones supported the preservation of stem and progenitor cells in the basal layer of the fibrin-based epithelial sheets, as demonstrated by multiple molecular stem cell markers (p63α, Bmi-1, K15, and ABCG2), whereas increased calcium concentrations and air-lifting induced terminal differentiation and gradual loss of stem cells.

CONCLUSIONS. The proposed culture system supports enrichment and survival of limbal stem and progenitor cells during the entire cultivation process and may be essential for long-term restoration of the damaged ocular surface. (Invest Ophthalmol Vis Sci. 2010;51:765–774) DOI:10.1167/iovs.09-4109

One emerging surgical strategy for restoring a normal corneal epithelial surface in patients with limbal stem cell (SC) deficiency is the transplantation of ex vivo expanded limbal epithelial SCs, one of the few adult human SC therapies currently being used.1−4 This therapeutic approach commonly involves the harvest of a small limbal biopsy from either the patient or a donor followed by cell expansion to generate an epithelial sheet on transplantable carriers, such as amniotic membranes,5−10 fibrin gels, and temperature-responsive polymers.11 Although successful repopulation of the ocular surface has been described for up to 1 year after transplantation, other studies indicate that epithelial viability is not sustained for longer periods12 and that there is no survival of donor cells beyond 9 months after transplantation.13,14 This failure may arise from the depletion of SCs in culture due to improper culture conditions. In most cases, the methods used to establish the cultures do not favor preservation of SCs (e.g., explant cultures or air-lift cultures), which promote proliferation and terminal differentiation of transient amplifying cells (TACs), but not retention of SCs.15 The long-term restoration of the damaged ocular surface, however, requires the preservation of limbal SCs during the culture process and after grafting.4,16

Since the pioneering work in 1975 by Rheinwald and Green,17 studies have shown that long-term survival and serial expansion of epithelial SCs are possible if the cells are cocultured with fibroblast feeder cells.18 Clonal analysis of human keratinocytes cultured on feeder layers has identified three types of clonogenic cells, giving rise to holoclones, meroclones, and paraclones.19 Holoclone-forming cells have all the hallmarks of SCs, including self-renewing capacity and a large proliferative potential, whereas meroclones and paraclones are generated by different stages of TACs with a limited capacity for proliferation. This discovery was followed by the identification of holoclone-forming cells in the limbal epithelium and the development of a culture system that involves enrichment of limbal SCs by clonal growth on a feeder layer before seeding onto fibrin gels to produce epithelial sheets.20,21 Consistently, keratinocytes cultured by this method have been used to permanently restore massive epidermal defects as well as the corneal surface of patients with total limbal SC deficiency.1,22−24 Nevertheless, the question of whether the transplanted cell sheets actually contain SCs has not been clarified, and the widespread use of this promising culture technique has been hampered by lack of a standardized cultivation protocol.

In this study, we evaluated the effect of several culture variables on clonal growth and preservation of SC phenotype in culture, to provide an optimized culture technique supporting preferential expansion and maintenance of limbal SCs for therapeutic applications. The results show that, by using clonal expansion and specific culture methods, sheets of epithelial cells that support preservation of an SC phenotype in their basal layers can be generated from small human limbal biopsy specimens. This proposed culture system may be essential for long-term clinical success and stability of the regenerated corneal epithelium.
MATERIALS AND METHODS

Cell Culture

Small biopsies (2 mm²) were obtained from the limbus of normal donor corneas (mean age, 72 ± 14 years; range, 58–86) subjected to corneal transplantation. Biopsies were taken from different areas of the limbus (posterior, inferior, temporal, and nasal). Single-cell suspensions were obtained by different enzymatic dissociation methods (described later) with a combined dispase II/trypsin-EDTA (ethylenediaminetetraacetic acid) treatment (procedure 1) used as the standard protocol. The cell suspensions were seeded at a density of 1 × 10^3 cells/cm² on a 3T3 feeder cell layer in six-well culture plates. The cell cultures were incubated at 37°C under 5% CO₂ and 95% humidity in different culture media (described later) with medium MCDB151 containing human corneal growth supplement (HCGS), 5 ng/mL human epidermal growth factor (EGF), 10% fetal calf serum (FCS), and 5 μg/mL gentamicin (medium 1) used as the standard medium.

Informed consent to limbal tissue donation was obtained from the relatives of the organ donors, and the research was conducted in compliance with the tenets of the Declaration of Helsinki for experiments involving human tissue.

Dissociation Methods. Four different methods of SC isolation from limbal tissue specimens were compared.

1. Specimens were incubated in dispase II solution (2.4 U/mL; Roche, Rotkreuz, Switzerland) for 1.5 hours at 37°C followed by incubation in 0.25% trypsin-0.02% EDTA (Invitrogen, Karlsruhe, Germany) for 10 minutes at 37°C, to obtain single-cell suspensions.

2. Specimens were minced and directly incubated in 0.25% trypsin-0.02% EDTA for 1.5 hours at 37°C followed by trypsinization-0.2% EDTA for 10 minutes at 37°C, to obtain single-cell suspensions.

3. Specimens were minced and directly incubated in 0.25% trypsin-0.02% EDTA for 1.5 hours at room temperature under continuous magnetic stirring at a speed of 500 to 700 rpm.

4. Specimens were treated with thermolysin (250 μg/mL; Sigma-Aldrich, Munich, Germany) for 10 minutes at 37°C followed by incubation in 0.25% trypsin-0.02% EDTA for 10 minutes at 37°C, to obtain single cell suspensions.

Culture Media. Five different culture media commonly used for the cultivation of epithelial SCs were compared.

1. MCDB151, containing 0.03 mM calcium supplemented with HCGS containing 0.18 μg/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL transferrin, 1 ng/mL EGF, and 0.2% bovine pituitary extract (Invitrogen).

2. Equal parts of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (DMEM/F12; Invitrogen), containing 1.2 mM calcium and supplemented with HCGS.

3. Defined keratinocyte serum-free medium (D-KSFM; Invitrogen) containing <0.1 mM calcium and D-KSFM supplement of unknown composition.

4. Progenitor cell targeting medium (PCT) CnT-20 (CellnTec, Bern, Switzerland) containing 0.07 mM calcium and CnT-20 supplement of unknown composition.

5. EpiLife medium (Invitrogen), containing 0.06 mM calcium supplemented with HCGS.

All media were supplemented with 5 μg/mL gentamicin (Invitrogen) and different concentrations (0%–20%) of FCS (Invitrogen) or EGF (0–20 ng/mL) (R&D Systems, Heidelberg, Germany). To evaluate the effect of further growth factors, serum-free MCDB151 medium was supplemented with one of the following growth factors, all at a concentration of 10 ng/mL (Millipore, Schwabach, Germany): transforming growth factor (TGF)-α, keratinocyte growth factor (KGF or FGF-7), platelet-derived growth factor (PDGF)-BB, leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), stem cell factor (SCF), insulin-like growth factor (IGF)-1, and fibroblast growth factor (FGF)-2. Neurotrophic factors, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), and artemin, all at a concentration of 100 ng/mL, were also included in the analysis.

Clonal Analysis and Growth Assay

Clonal expansion of limbal stem and progenitor cells was performed by seeding single-cell suspensions at a density of 1 × 10^5 cells/cm² on a mitomycin C-treated 3T3 feeder layer, as previously described.25 Colony formation was monitored daily by phase contrast microscopy and analyzed on day 14 after removal of the feeder layer with 0.02% EDTA (Sigma-Aldrich) for 30 seconds, fixation in 4% paraformaldehyde and staining with 2% rhodamine B (Sigma-Aldrich). The colonies were classified into holo-, mero-, and paracolones, according to their size, morphology, number of cells, and cellular phenotype, as originally reported.19 The number of cells of individual holoclines was determined by using cloning cylinders (Sigma-Aldrich) and a cell-counting system (CASY; Roche Innovatis, Basel, Switzerland). Colony size, defined as diameter of individual holoclines in millimeters, and colony density, defined as the percentage area of the culture dish covered by all holoclines, were determined by using image-analysis software (CellF; Olympus, Hamburg, Germany). Colony-forming efficiency (CFE) was calculated as the number of holo-, mero-, and paracolones divided by the total number of cells seeded per well. For serial propagation, the clonal cells were passaged after 14 days of culture, seeded at a density of 1 × 10^3 cells/cm² on a 3T3 feeder layer, and cultured for another 14 days.

Statistics

Data are presented as the mean ± SD. Statistical evaluation of significant differences between culture methods was performed with the Mann-Whitney test for nonparametric analysis. P < 0.05 was considered statistically significant.

Phenotypic Characterization of Colonies

For immunocytochemistry, limbal epithelial cells were cultured for approximately 2 weeks either in glass chamber slides (LabTek; Nunc, Wiesbaden, Germany) or on fibrin gels together with a 3T3 feeder layer and processed as previously described.25 Immunocytochemical staining was performed with primary antibodies (Table 1) diluted in PBS. Antibody binding was detected by Alexa 488- or Alexa 555-conjugated secondary antibodies (Invitrogen-Molecular Probes, Germany), and nuclear counterstaining was performed with propidium iodide or DAPI (4’,6’-diamino-2-phenylindole; Sigma-Aldrich). In negative control experiments, the primary antibody was replaced by PBS or equimolar concentrations of an irrelevant primary antibody.

For light and transmission electron microscopy, the colonies were processed as previously described.25

Subculture of Clonally Expanded Cells on Fibrin

Fibrin gels were prepared as previously described.25 Limbal epithelial holoclines were harvested by trypsinization (0.25% trypsin-EDTA for 15 minutes at 37°C) after approximately 21 days, seeded on top of fibrin gels at a density of 1 × 10^5 cells/cm², and subcultivated in MCDB151 medium supplemented with HCGS, 5 ng/mL EGF, 100 ng/mL β-NGF, 10% FCS, and 5 μg/mL gentamicin for a further 14 to 16 days. Culture conditions were switched from low-calcium conditions (0.03 mM Ca^{2+}) to medium-calcium conditions (0.4 mM Ca^{2+}) by adding CaCl₂ (Sigma-Aldrich) to the MCDB151 medium after 7 days, to promote the formation of adhesion molecules. For comparative analyses, additional experiments were performed by using high-calcium culture conditions (1.2 mM Ca^{2+}). The fibrin gels were finally fixed and processed for paraffin embedding, transmission electron microscopy, and immunohistochemistry.25
RESULTS

Clonal Growth and Phenotypic Characterization

When supported by growth-arrested 3T3 feeder cells, human limbal epithelial cells gave rise to macroscopic colonies within 10 to 12 days after inoculation. Average CFE ranged from 0.3% to 0.4%. After 3 to 4 weeks, the colonies eventually fused and generated a stratified layer. The colonies were classified into hol-, mero-, and paraclones according to their size, morphology, number of cells, and cellular phenotype, after 2 weeks of culture in MCDB151 medium supplemented with HCGS, 10% FCS, and 5 ng/mL EGF. In general, large, nearly circular colonies (4–10 mm in diameter) with smooth outlines were identified as holoclones, and small, medium-sized colonies (1–4 mm in diameter) with wrinkled outlines as meroclones, and small, identified as holoclones, medium-sized colonies (1–4 mm in diameter) with smooth outlines were identified as meroclones, and small, highly irregular colonies (<1 mm in diameter) as paraclones. The holoclones contained 2 to 5 × 10⁴ cells/mm² and consisted mainly of small cuboid, densely packed cells in a regular mosaic pattern, concentrated in the periphery of the colony (Fig. 1A). The center of the colony appeared stratified, with the upper differentiating layers consisting of large, flattened cells covering the basal layer of small cuboid cells. In contrast, most cells in the meroclones and all cells in the paraclones had an increased cell size and appeared flattened (Figs. 1B, 1C, respectively). Immunocytochemical analysis of whole holoclones with antibodies against suggested limbal progenitor (p63, p63/SC-specific isoform of p63) and marker for holoclone identification, was restricted to small cells in the periphery (Fig. 2C). There was almost no overlap between desmoglein-3+ and p63α+ cells. Expression of Bmi-1 in peripheral cells (Fig. 2D) has been also reported as a marker of human limbal holoclones. Expression of ABCG2 was observed in clusters of cells close to the clone border (Fig. 2E). Each holoclone contained one to few cells in the periphery, which showed cytoplasmic labeling for K15 and Notch-1 (Fig. 2G). These cells were characterized by a small diameter <10 μm and a large nuclear–cytoplasmic ratio, thus potentially representing stem or progenitor cells. Cells positive for K19 were distributed throughout the basal layer of the colonies (Fig. 2H). K3/K12 immunostaining, indicative of the extent of corneal epithelial differentiation, was confined to a few superficial cells in the central stratified region (Fig. 2I).

Effect of Limbal Region and Donor Age on Clonal Growth

To evaluate the clonogenic ability of the different areas of the limbus, cells from the superior, inferior, nasal, and temporal limbus of six donor eyes (69 ± 17.1 years) were analyzed. The CFE was comparable in the superior and inferior as well as in the nasal and temporal regions and was markedly higher in the superior (0.4%) and inferior (0.3%) than in the nasal (0.1%) and temporal (0.1%) areas of the limbus. Consistently, colony density and colony size were significantly larger in the superior and inferior regions than in the nasal and temporal regions (Fig. 3.1). Based on these observations, the superior...
limbus was selected as the region of tissue sampling for all further experiments.

The average CFE declined with the age of the donor. In patients aged 50 to 65 years ($n=11005$), the CFE was 0.5% to 0.6%, but the CFE dropped significantly to 0.3% in patients aged 65 to 85 years ($n=6005$). Colony density and size decreased from 80% and 8 mm diameter to 40% and 4 mm diameter in the older age group (data not shown).

**Effect of the Dissociation Method on Clonal Growth**

To establish the most effective method of SC isolation from small biopsies (2 mm$^2$) taken from the superior limbus of four pairs of donor eyes (59 ± 15.7 years), four different enzymatic dissociation methods were compared. Although the total cell yield was comparable between all methods used, amounting to approximately $1 \times 10^4$ cells/mm$^2$ tissue, the CFE showed significant differences between dispase II/trypsin-EDTA (0.37%), thermolysin/trypsin-EDTA (0.01%), trypsin-EDTA with magnetic stirring (0.35%), and trypsin-EDTA with agitation (0.34%). Colony density and size were highest with a combined extraction method using dispase II/trypsin-EDTA and a single extraction method using trypsin-EDTA with agitation ($P < 0.05$), although the coefficient of variation was greater when the latter method was used, because of the highly variable proportions of live and dead cells in the single-cell suspensions (Fig. 3.2). Therefore, the combined dispase II/trypsin-EDTA digestion method, which consistently yielded a high number of viable cells (85%-90%), was selected as the standard isolation procedure for all further experiments.

**Effect of Culture Conditions on Clonal Growth**

To evaluate the effect of culture conditions on clonal growth, we first compared five different culture media containing low (MCDB151, PCT, D-KSFM, and EpiLife) or high (DMEM/F12) calcium concentrations, commonly used for ex vivo expansion of corneal and epithelial progenitor cells. Although the total...
CFE was comparable in all media (0.37% in MCDB151, 0.35% in PCT, 0.31% in D-KSFM, and 0.4% in DMEM/F12) except for EpiLife, colony growth was fastest in DMEM/F12 and slowest in MCDB151, whereas EpiLife did not support clonal growth at all. Consistently, cells cultured in DMEM/F12 showed a significantly higher colony density and size than those grown in MCDB151 after 2 weeks of culture ($P < 0.05$; Fig. 4). However, these fast-growing colonies mostly consisted of large, flattened, apparently differentiated cells that reached senescence after 3 to 4 passages. Similarly, the colonies grown in PCT and D-KSFM revealed a rather irregular outline and pleomorphic cellular phenotype (Fig. 4). Despite delayed growth in MCDB151, this medium produced the most regular and compact holoclones consisting of small, tightly packed cells that could be serially cultured for at least 8 to 10 passages. A prolonged culture time of 3 to 4 weeks promoted further clonal growth in MCDB151 up to complete overgrowth of the culture plate. Therefore, MCDB151 was selected as the most effective culture medium to support the growth of limbal holoclones and used as the standard medium for all further experiments.

Addition of 10% FCS and 5 ng/mL EGF to the culture medium resulted in a significantly increased colony density and size when compared with lower or higher concentrations of serum and growth factors (Figs. 5A–D). Formation of holoclones was largely inhibited in serum-free medium, but addition of growth factors resulted in the formation of small rosettelike cell clusters, amenable to quantitative analysis. Comparison of EGF with other growth factors under serum-free culture conditions showed that both TGF-α and KGF comparably stimulated clonal growth, whereas all other growth factors tested were significantly less effective (Fig. 5E). Supplementation with neurotrophic factors had no effect on clonal growth, but β-NGF showed an additive effect, with EGF resulting in a threefold increased growth rate compared with EGF alone (Fig. 5F).

**Effect of Culture Conditions on Clonal Cell Phenotype**

Since extracellular calcium concentration is a decisive factor that modulates epithelial growth and differentiation,29,30 we compared the effects of culture media with particularly low (MCDB151) and high (DMEM/F12) calcium concentrations on the cellular phenotype. Cells in MCDB151-cultured clones were uniformly smaller and expressed lower levels of K3/K12 but higher levels of progenitor cell markers, such as ABCG2.
and K15, when compared with cells cultured in DMEM/F12 (Figs. 6A–D). P63α, as an SC determinant in holoclones, was markedly expressed under low calcium conditions but considerably declined under high calcium conditions (Figs. 6E, 6F).

Transmission electron micrographs of vertical sections through DMEM/F12-cultured colonies revealed a stratified epithelial layer composed of elongated, largely differentiated cells containing apical microvilli, prominent cytoplasmic filaments, and abundant desmosomes in all layers. In contrast, colonies grown in MCDB151 showed a basal layer of small, rather undifferentiated cuboid cells centrally covered by one to two layers of elongated cells with beginning signs of epithelial differentiation (Figs. 6G, 6H).

**Generation of Epithelial Cell Sheets on Fibrin**

After the holoclones were dissociated and transferred and before adjacent colonies merged (i.e., 21 days on average), clonally expanded limbal progenitor cells were seeded onto fibrin gels. Subcultivation of cells in MCDB151 medium supplemented with 10% FCS and 5 ng/mL EGF, which was switched from low calcium (0.03 mM) to medium-level calcium (0.4 mM) concentrations after 7 days, gave rise to stratified cohesive epithelial cell sheets consisting of a basal layer formed by cuboid cells and two to three suprabasal layers of elongated cells, after 14 to 16 days in submersin culture (Figs. 7A, 7C). Moderate K3/K12 expression was restricted to superficial cell layers (Fig. 7E), whereas positive staining for p63α, Bmi-1, ABCG2, and K15 was restricted to clusters of mainly basally located cells (Figs. 7G, 7I, 7K), indicating preservation of the SC phenotype within the epithelial construct. After dissociation of cells from the fibrin substrate, CFE was calculated as 0.15%. Elevation of calcium concentration to 1.2 mM considerably declined under high calcium conditions (Figs. 6E, 6F). Transmission electron micrographs of vertical sections of pleomorphic cells in PCT (D) and DMEM/F12 (E) showed significantly increased clonal growth in DMEM/F12 compared with that in MCDB151. Values represent the mean ± SD of results in five separate experiments (\(^{*} P < 0.05;\) Mann-Whitney test).

**DISCUSSION**

Transplantation of ex vivo expanded limbal epithelial cells has become a routine treatment for ocular surface reconstruction in patients with limbal SC deficiency in several clinical centers. However, the widespread use of cultured limbal epithelial autografts has been hampered by different techniques of cultivation and the variable clinical results reported. Differences in culture techniques include the use of explant or single-cell suspension systems; the presence or absence of a 3T3 feeder layer; the use of different carriers, including fibrin and amniotic membrane; and the use of air-lifting to promote epithelial differentiation and stratification. The most widely used method is the explant culture system, in which a small limbal biopsy is placed on the carrier, and the limbal epithelial cells then migrate out of the biopsy and proliferate to form an epithelial sheet. However, outgrowths from human limbal explants show a rapid decline in proliferative potential and it is assumed that TACs rather than SCs actually migrate onto the culture substrate. The use of limbal epithelial cell suspensions instead of limbal explants may increase the proportion of SCs in the culture system. Nevertheless, it is still unclear whether cultivated epithelial sheets contain sufficient amounts of limbal stem and progenitor cells, which is the key to ensuring successful and long-term regeneration of the ocular surface.

In this study, we determined the optimal method for the expansion of epithelial SCs derived from a small limbal biopsy, which greatly increases the survival of stem and progenitor cells in culture. The system uses clonal enrichment of limbal SCs and their subcultivation on fibrin as a transplantable carrier. It is based on the pioneering work of Rheinwald and Green, who showed in 1975 that human epidermal SCs can be expanded by clonal growth on a fibroblast feeder layer, which was later adapted to the amplification of limbal SCs by Pellegrini et al. Subsequently, cultivated epithelial sheets seeded with clonally enriched stem and progenitor cells have been used as epithelial autografts for long-term skin replacement and ocular surface reconstruction, and demonstration of holoclones within epithelial grafts has been consid-
ered a means of quality control. However, it has been stressed that improper culture conditions can irreversibly induce clonal conversion and hence cause a rapid disappearance of SCs, strengthening the importance of a thorough evaluation of the impact of cultivation procedures on SC behavior and phenotype.

In this study, we used analyses of clonogenic capacity and clonal growth, measured by CFE, colony size, colony density, and proliferative potential, to objectively evaluate the effects of culture variables and to screen for agents with growth-promoting activities. For definition of clonal types, we applied a direct and practical classification, which is based on morphologic criteria, but not on the percentage of abortive colonies as originally described by Barrandon and Green. The findings provide evidence that the efficacy of SC isolation is highest using biopsies from the superior limbus, which showed the greatest number of holoclones and a combined dispase II/trypsin-EDTA enzymatic dissociation method. It has been suggested previously that corneal SCs are not evenly distributed throughout the human limbus, being more abundant in the inferior and superior regions than in the nasal and temporal regions, although others have reported on a higher CFE in the superior and temporal quadrants. The precise sampling location is particularly relevant, when limited amounts of tissue are available for SC isolation and expansion. We have demonstrated in this study that an initial biopsy of 2 mm² taken from the superior limbus of a 50-year-old donor yielded approximately 2 × 10⁶ cells and could be clonally expanded in vitro to 1 to 2 × 10⁶ cells. However, the CFE declined with the age of the donor, which is in consistency with previous reports.

Clonal growth and preservation of SCs require both the presence of a 3T3 feeder layer and proper serum concentrations. Furthermore, addition of growth factors, most importantly EGF or TGFα, to the culture medium has become a standard requisite. In this study, 10% FCS was the optimum concentration to stimulate CFE and colony growth. The clonogenic potential was further increased by addition of 5 ng/mL EGF to the medium, but equal concentrations of TGFβ or KGF were found to be similarly effective to increase the clonal growth rate. KGF is highly expressed by limbal fibroblasts, suggesting that KGF may be involved in...
modulating limbal SC function.\textsuperscript{45} However, cells grown in the presence of both EGF and β-NGF showed the highest rate of colony expansion, which was increased approximately threefold compared with EGF alone. NGF has been associated with the proliferation and differentiation of human corneal epithelial cells.\textsuperscript{46} Its high-affinity receptor TrkA is expressed in both corneal and limbal epithelial basal cells suggesting that NGF signaling favors limbal SC survival in vivo.\textsuperscript{47} Blocking NGF signaling significantly retarded cell expansion on amniotic membrane supporting the notion that NGF is also important for the expansion of limbal epithelial progenitor cells in vitro.\textsuperscript{48}

Long-term preservation and proliferation of SCs and their progenitors was supported by maintaining holoclones in a growth medium (MCDB151 supplemented with HCGS, 10% FCS and 5 ng/mL EGF) containing extremely low calcium concentrations (0.03 mM). It is well known that increasing concentrations of extracellular calcium induce epithelial cell differentiation.\textsuperscript{29,30,49–51} Consistently, clonal cells grown in MCDB151 showed an undifferentiated phenotype and could be serially cultured for at least 8 passages, whereas the rather differentiated cells of the fast-growing colonies in DMEM/F12 (1.2 mM calcium) reached senescence after 3 to 4 passages.

Low calcium concentrations were not only used for clonal expansion, but, together with appropriate serum (10% FCS) and growth factor (5 ng/mL EGF and 100 ng/mL NGF) concentrations, were also used in the initial phase of subcultivation of clonally enriched cells on fibrin gels. By the dissociation and transfer of primary holoclones before adjacent colonies merged, the subcultured cell population became enriched with proliferative progenitor cells. Fibrin was selected as a suitable substrate, because it has been shown to support cell proliferation and to be degraded within 24 hours after transplantation.\textsuperscript{21} However, culture conditions were switched to medium-level calcium concentrations (0.4 mM) after 7 days, to promote the development of proper cell adhesions required for establishing a multilayered epithelial cell sheet and to simultaneously allow maintenance of stem and progenitor cells within its basal layer.

This proposed culture protocol is supposed to support the survival of limbal stem and progenitor cells during the entire cultivation process, as demonstrated by immunohistochemical verification of acknowledged molecular SC markers, such as p63α, Bmi-1, ABCG2, and K15 in both holoclones and the basal layer of multilayered epithelial cell sheets. Although the presence of holoclones in cultured limbal grafts has been evaluated in a previous study,\textsuperscript{22} this is the first study to demonstrate the preservation of SC in a fibrin-based epithelial construct by using multiple molecular SC markers. Immunodetection of ΔNp63α and Bmi-1 has been described as an important method for identification of SC-derived holoclones and the presence of SC within a cultured graft.\textsuperscript{1,4,16,22,26,28,52} Analysis of the CFE of cells dissociated from the fibrin matrix indicated the persistence of SCs at a percentage of 0.15% of the total number of cells. However, increasing the calcium concentration to 1.2 mM induced gradual loss of progenitor cells, as reflected by a marked decline of p63α, Bmi-1 and K15 staining and an increase in K3/K12 expression. Thus, the commonly applied culture methods using high calcium concentrations and air-lifting, do not favor preservation of SCs, but promote terminal differentiation of TAGs. However, the proposed submersion culture system using low- to medium-level calcium concentrations, appropriate serum concentrations and growth factor combinations, can be used to reconstitute transplantable cohesive sheets of partly differentiated epithelial cells retaining undifferentiated stem and progenitor cells in the basal layer.

In conclusion, we propose an improved culture protocol using biopsies from the superior limbus, a gentle two-step enzymatic dissociation method, clonal expansion of isolated SCs followed by subcultivation of holoclones on fibrin in a defined environment, supporting the preservation of stem and progenitor cells during the culture process. Whether this culture technique in fact enhances the therapeutic potential of limbal SC transplantation remains to be evaluated. Nevertheless, this culture system may represent a starting point for establishing a true SC-based therapy for long-term ocular surface reconstruction, which may be further improved for clinical use (e.g., by excluding xenobiotic products), such as FCS and 3T3 feeder cells, from the culture system.\textsuperscript{53} Moreover, for an extended survival of SCs in the cultured graft, factors repro-

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**FIGURE 6.** Effect of extracellular calcium concentration on clonal cell phenotype. (A–F) Comparative immunofluorescence analysis of holoclones expanded in culture medium with particularly low (MCDB151) and high (DMEM/F12) calcium concentrations using antibodies against SCs (ABCG2, p63α) and differentiation (K3/K12) markers. Cells in MCDB151-cultured clones expressed lower levels of K3/K12 (A) but higher levels of ABCG2 (C) and p63α (E) when compared to cells cultured in DMEM/F12 (B, D, F). (G, H) Transmission electron micrographs of vertical sections through holoclones expanded in MCDB151 revealed a basal layer of small, rather undifferentiated cuboid cells covered by one to two layers of elongated cells (G), whereas holoclones grown in DMEM/F12 showed a stratified epithelial layer composed of elongated, flattened, and largely differentiated cells (H). Nuclear staining was performed with propidium iodide (red, A–D) or DAPI (‘, 6-diamidino-2-phenylindole; blue, E, F). Magnification: (A, B) ×20; (C, D) ×100; (E, F) ×40.
FIGURE 7. Generation of epithelial cell sheets on fibrin gel. (A–L) Comparative phenotypic analysis of clonally expanded epithelial cells subcultivated on fibrin in media containing low to medium (MCDB151) or high (DMEM/F12) calcium concentrations for 14 to 16 days in submersion culture. (A–D) Light (A, B) and electron (C, D) microscopic analyses showed multilayered epithelial cell sheets consisting of a basal layer formed by cuboid cells and two to three suprabasal layers of elongated cells in MCDB151 (A, C), but a stratified epithelial sheet composed of five to six layers of elongated, largely differentiated cells in DMEM/F12 (B, D). Immunofluorescence analysis (E–L) showed that K3/K12 expression was restricted to superficial cell layers (E), whereas positive staining for p63α (G), Bmi-1 (I), and K15 (K) was observed in clusters of mainly basally located cells, when MCDB151 was used as culture medium. In contrast, cultivation in DMEM/F12 caused a pronounced increase in K3/K12 expression (F) and a marked decline in expression of p63α (H), Bmi-1 (J), and K15 (L). Staining: periodic acid–Schiff (A), hematoxylin-eosin (B). Nuclear staining was performed with propidium iodide (red, E, F, I–L) or DAPI (4′,6-diamidino-2-phenylindole; blue, G, H). Magnification: (A, B) ×40; (E–L) ×100.

producing several aspects of the niche environment have to be integrated into the culture system in the future.

References


