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Presence of native limbal stromal cells increases the expansion efficiency of limbal stem/progenitor cells in culture

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Abstract

Niche factors are important in the maintenance and regulation of stem cells. Limbal stromal cells are potentially a component of limbal stem cell (LSC) niche. We investigated the role of the limbal stromal cells in the *ex vivo* expansion of limbal stem/progenitor cells. Limbal epithelial cells were cultured as single-cell suspension and cell clusters from dispase II or collagenase A (ColA), or tissue explant. ColA isolated limbal stromal cells along with limbal epithelial cells. In the presence of limbal stromal cells, a higher absolute number of $p63\alpha^{bright}$ cells ($p < 0.05$) and a higher proportion of K14 positive epithelial cells were obtained from both ColA and explant tissue cultures. Expansion of the stem/progenitor population from dispase isolation was more efficient in the form of cell clusters than single cell suspension based on the absolute number of $p63\alpha^{bright}$ cells. Expansion of the stem cell population is similar in the single cell and cell cluster cultures that are derived from ColA isolation. Our finding suggests that limbal stromal cells and an intact cell–cell contact help to maintain LSCs in an undifferentiated state *in vitro* during expansion.

Keywords

limbal epithelial cells; limbal stromal cells; dispase II; collagenase A; explant culture; limbal stem cell niche

1. Introduction

The corneal epithelium is continuously regenerated by corneal epithelial stem/progenitor cells (limbal stem cells; LSCs) located at the limbus. The specific location and niche of LSCs are thought to be at the limbal epithelial crypt or lacuna deeper in the limbal stromal (Dua et al., 2005; Shortt et al., 2007; Zarei-Ghanavati et al., 2011). Like other stem cells, LSCs are small and have a high nuclear-to-cytoplasm ratio. They are slow cycling *in vivo* but have a high proliferation rate in response to injury and in culture. LSCs lack the expression of cytokeratin (K) 3 and K12 of differentiated corneal epithelial cells and express several putative stem cell markers including ATP-binding cassette sub-family G member 2 (ABCG2; Watanabe et al., 2004), ΔNp63α (Pellegrini et al., 2001), K14 (Barnard et al., 2001), K15 (Yoshida et al., 2006) and N-cadherin (Hayashi et al., 2007).

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Appendix A. Supplementary data: Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2013.08.020.

Although the location and the components of the LSC niche are largely unknown, this niche is thought to possess unique properties that provide a special microenvironment to maintain LSCs in a stemness state (Li et al., 2007). Crosstalk among niche cells, extracellular matrix components, and soluble factors controls the homeostasis and differentiation cues to replenish the cornea epithelium. Identification and characterization of all these mechanisms will help to establish the optimal conditions to isolate, expand, and maintain these cells in an undifferentiated state *in vitro*.

Damage of the limbus leads to limbal stem cell deficiency (LSCD), which is characterized by the inability to regenerate the corneal epithelium (Secker and Daniels, 2008; Tseng, 1989; Zarei-Ghanavati and Deng, 2013). Cornea transplantation is ineffective, so the restoration of the LSC population is the only effective treatment. Transplantation of *ex vivo* expanded autologous LSCs to treat unilateral LSCD would be a preferred method of restoration because only a small amount of the donor tissue is needed.

The quality of the *ex vivo* expanded LSCs may be influenced by many factors including the source of the cells, the method of isolation from the tissue and the culture conditions such as the substrate, feeder cells and culture medium. Many isolation methods have been tested and compared. Some groups have used the chelating agent ethylenediaminetetraacetic acid (EDTA; Spurr and Gipson, 1985) and the trypsin enzyme (Kim et al., 2004; Meyer-Blazejewska et al., 2010). Others have isolated the LSCs using the conventional enzymatic digestion with dispase II (Espana et al., 2003; Kim et al., 2004, 2006; Koizumi et al., 2002; Meyer-Blazejewska et al., 2010; Zhang et al., 2005). More recently, Chen et al. (2011) reported for the first time the novel collagenase A (ColA) isolation method which can isolate all LSCs in combination with the immediate underlying stromal cells. Many other groups have tested the explant culture (Basu et al., 2012; Ghoubay-Benallaoua et al., 2011; Kim et al., 2004; Koizumi et al., 2002; Zhang et al., 2005). Moreover, it has been shown that cultures that contain a minimum of 3% of $p63\alpha^{bright}$ ($p63\alpha^{br}$) stem/progenitor cells could achieve a transplantation success of the 78% (Rama et al., 2010). This result suggests that a minimal number of stem/progenitor cells must be produced in culture to repopulate the corneal surface.

In the present study, we investigate whether the presence of limbal stromal cells affected the LSC *ex vivo* expansion. To address this question we compare the cultures that contain both LSCs and the limbal stromal cells including the explant culture and the cultures of LSCs isolated using ColA (ColA culture) to the cultures of LSCs isolated using dispase (dispase culture) which only isolates LSCs. To our knowledge, this is the first study comparing the three culture methods in parallel to determine the most efficient LSC *ex vivo* expansion method. Our results indicate that the presence of limbal stromal cells in the culture helps to maintain the LSC phenotype.

2. Material & methods

2.1. Human tisclerocorneal tissue

Human sclerocorneal tissue was obtained from Illinois Eye Bank (Watson Gailey, Bloomington, IL) and Lions Eye Institute for Transplant and Research (Tampa, FL) from 20 to 65 years old healthy donors. Human tissue was handled in accordance with the tenets of the Declaration of Helsinki. The experimental protocol was exempted by the University of California Los Angeles Institutional Review Board.

The tissues were preserved in Optisol™ (Chiron Ophthalmics, Inc., Irvine, CA) at 4 °C for less than 72 hours (h). For cell culture, the death-to-preservation time was less than 8 h. The

sclerocorneal rim was collected immediately after cornea transplantation and processed no later than 2 days.

2.2. Limbal epithelial cells isolation and explant culture

After removal of the iris, endothelium, conjunctiva and Tenon's capsule, the sclerocorneal rim tissue was cut into 3 segments to perform the dispase and ColA incubation and to cut the limbal explant pieces to eliminate tissue variability from at least three different donors. An epithelial cell sheet was isolated from the limbal rim by incubation with 2.5 mg/mL of dispase (Roche, Indianapolis, IN) at 37 °C for 2 h or by incubation with 1 mg/mL of ColA in DMEM/F-12 (Ham) medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Invitrogen). Four sets of conditions for the ColA incubation were tested after the sclerocorneal rim tissue was cut into 4 equal segments from three different donors: at 37 °C overnight (ON) in the incubator (37 °C ON INC), at 4 °C ON (4 °C ON), at 37 °C for 2 h in the water bath (37 °C 2 h WB) and at 37 °C for 2 h in the incubator (37 °C 2 h INC). The most efficient ColA incubation was chosen to be compared to the dispase treatment and the explant culture. After the cell sheet from both dispase and ColA treatments was removed from the tissue, it was either incubated with 0.25% trypsin – 1 mM EDTA (Invitrogen) to achieve a single-cell suspension or pipetted multiple times to break the cell sheet into small cell clusters. For the explant culture, a 2×2 mm limbal tissue was used and half of the sclera was removed.

2.3. Cell cultures

Limbal epithelial cells and explants were cultured in SHEM5 medium consisting of DMEM/F-12 (Ham) medium that included N2 supplement (Invitrogen), 2 ng/mL of epidermal growth factor (Invitrogen), 8.4 ng/mL of cholera toxin (Sigma–Aldrich, St. Louis, MO), 0.5 g/mL of hydrocortisone (Sigma–Aldrich), 0.5% of dimethyl sulfoxide (Sigma–Aldrich), and 5% FBS at 37 °C under 5% CO_2. The medium was changed every 2–3 days.

3T3-J2 mouse fibroblasts (Howard Green Lab, Harvard Medical School) that had been growth arrested with mitomycin C (Sigma– Aldrich) were seeded at a density of 3×10^4 cells/cm^2 in 6-well plates. Limbal epithelial cells (300 cells/cm^2) were seeded onto the 3T3 cells for both dispase and ColA isolation. To obtain a more accurate cell density, a small portion of the cell clusters were trypsinized to achieve single cell suspension. The proportion of LSCs seeded was adjusted accordingly to make both treatments comparable based on the amount of LSCs isolated by each treatment.

To assess the colony forming efficiency (CFE), colonies on culture plates were fixed with 4% paraformaldehyde (PFA) and stained with rhodamine B solution (Sigma–Aldrich). Colonies were counted by using the Image J software (Bethesda, MD). The calculation of CFE was based on the number of LSC colonies divided by the number of LSCs seeded. The presence of holoclones, defined as round and compact colonies with small cells both in the center and periphery (Barrandon and Green, 1987), was analyzed in the cultures. CFE was performed in dispase and ColA-isolated single cell cultures.

The analysis of the cell growth rate was based on the number of cells collected by the end of the culture divided by the number of cells seeded. For the explant culture, an approximation of the number of LSCs isolated in each 2×2 mm limbal tissue was used.

2.4. Quantitative RT-PCR

LSC colonies were isolated after washing away the 3T3 cells by pipetting the media up and down to break the 3T3 cells monolayer. The LSC colonies were lysed and homogenized by using a shredding system (QIAshredder; Qiagen, Valencia, CA). Total RNA of LSCs was

extracted by using RNeasy Mini Kit (Qiagen). The quantity and quality of total RNA were assessed with a spectrophotometer (NanoDrop 1000; NanoDrop, Wilmington, DE). Total RNA with minimal degradation was subjected to DNase treatment (Ambion INC, Austin, TX) according to the manufacturer's instruction.

Total RNA was reverse-transcribed (Superscript II RNase H2 reverse transcriptase; Invitrogen) according to the manufacturer's recommendations. The relative abundance of transcripts was detected by quantitative (q)RT-PCR (Brilliant SYBR Green Master Mix, Mx3000p real-time PCR system; Stratagene, La Jolla, CA). Cycling conditions were as previously reported (Nakatsu et al., 2011). The fluorescence intensity of each sample was normalized in relation to that of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The average value of triplicates from each transcript was used for comparison. Quantitative RT-PCR was conducted independently three times with samples from each donor. The primers used for qRT-PCR are listed in Supplementary Table S1.

2.5. Immunofluorescent staining

Cytospin slides were prepared by using a cytocentrifuge (Cytofuge; Fisher Scientific) and subsequently stored at −20 °C. Tissue sections from human sclerocorneal tissues were embedded in OCT (Sakura Finetek, Torrance, CA) on dry ice. Tissues were cut in 8 μm cryosections by a cryostat (Leica CM3050S; Leica Microsystems, Wetzlar, Germany) and stored at −80 °C until they were processed.

Cytospin slides and tissue sections were processed as previously reported (Nakatsu et al., 2011). Pictures were taken with a confocal microscope (Confocal Laser Scanning Microscopy; Olympus, San Jose, CA) and an image capture and analysis system (Fluoview FV10-ASW 3.1 Viewer; Olympus). Primary antibodies that were used are listed in Supplementary Table S2. The number of K14, K12 and vimentin (Vim) positive cells was calculated by using Image J software.

2.6. Quantification of p63α^{br} cells

To calculate the percentage of p63α^{br} cells, the nuclear intensity of p63α was measured based on the criteria previously reported (Di Iorio et al., 2006) using the Definiens Tissue Studio software (Larchmont, NY). The absolute number of p63α^{br} cells in culture was calculated.

2.7. Statistical analysis

Data were analyzed by using the Student's *t*-test. Graph bars are expressed as the mean ± standard error of the mean (SEM) from at least three separate experiments. Values with a $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Optimization of the ColA treatment to maximize the isolation of LSCs

Before comparing the LSC *ex vivo* expansion among dispase, ColA and explant cultures, four different ColA isolation conditions were tested to optimize the yield of limbal stromal cells and LSCs.

The ColA 37 °C 2 h INC treatment was the most efficient method to isolate maximal number of K14^{+}/Vim$^{−}$ epithelial cells ($3.12 \pm 0.04 \times 10^{5}$) and adequate number of K14$^{−}$/Vim^{+} stromal cells ($3.49 \pm 0.51 \times 10^{4}$; Supplementary Fig. S1A–E). This isolation method also generated the best holoclone morphology in culture (Fig. 1D) and the cultured LSCs expressed high levels of ABCG2, ΔNp63 and K14 and low levels of K12 (Fig. 1E).

The ColA 37 °C 2 h INC method was chosen for comparison with dispase isolation and explant culture in the rest of experiments. LSC cultures derived from the ColA isolation method are termed “ColA cultures” and the ones from the dispase isolation method are termed “dispase cultures”.

3.2. Comparison of the limbal epithelial cells isolated by dispase II and ColA digestion

To further analyze the cell population isolated by using ColA and dispase, limbal tissues after digestion were sectioned and stained for K14, p63α and the stromal markers Vim and tenascin C (TNC). As expected, K14 and p63α were expressed in the basal and suprabasal layers of the limbus. Vim and TNC were expressed in the limbal basement membrane and stroma (Ding et al., 2008; Maseruka et al., 2000; Schlotzer-Schrehardt et al., 2007) and Vim was also observed in a small fraction of the basal limbal cells as previously reported (Chen et al., 2011; Schlotzer-Schrehardt and Kruse, 2005; Supplementary Fig. S2A–C).

After dispase digestion, some $K14^+$ and $p63\alpha^+$ clusters surrounded by Vim^+ cells remained in the deep limbal tissue (Supplementary Fig. S2D and E, arrow). These $K14^+$ cells co-localized with TNC in the basement membrane (Supplementary Fig. S2F, arrow). However, after ColA incubation, we did not detect $K14^+$, $p63\alpha^+$ or TNC^+ cells (Supplementary Fig. S2G–I). It is noteworthy that the tissue structure was better preserved after dispase incubation; this finding indicates more efficient digestion of limbal collagen tissue by ColA to release deeply seeded basal epithelial cells as previously reported (Chen et al., 2011).

The phenotype of the freshly isolated cells by the ColA was characterized by a slightly higher expression of putative LSC markers. K12 expression was lower and α-SMA expression was higher in the cells isolated by ColA than by dispase, probably as a result of the higher number of stromal cells obtained by the ColA than by the dispase (Supplementary Fig. S2J). Double immunostaining for K14/Vim revealed a larger amount of $K14^+$ epithelial cells isolated by ColA ($3.12 \pm 0.04 \times 10^5$) than by dispase ($2.1 \pm 0.2 \times 10^5$, $p = 0.02$). ColA resulted in the isolation of 5.8 fold more of Vim^+ cells ($3.5 \pm 0.5 \times 10^4$) than dispase ($6.0 \pm 1.9 \times 10^3$, $p = 0.009$; Table 1).

3.3. Comparison of LSC ex vivo expansion by different culture methods

ColA appeared to be superior to dispase in its ability to isolate limbal basal epithelial cells and adjacent stromal cells as previously reported (Chen et al., 2011). We next compared the growth of the LSC population from the dispase, ColA and explant cultures on 3T3 cells. All three culture systems supported the growth of limbal epithelial cells with stem/progenitor morphology (Fig. 2A–D). Collagenase-isolated single cells generated a higher CFE than dispase-isolated single cells (3.7 ± 0.5 and 1.7 ± 0.3, respectively, $p = 0.04$; Fig. 2E) and a higher cell growth rate (Fig. 2F).

The LSCs grown in the ColA culture expressed a higher mRNA level of ΔNp63 and a lower level of K12 than LSCs from dispase or explant cultures (Fig. 3A). Except for ABCG2 which is lower in the explant culture, the expression of all other putative LSC markers from ColA and explant cultures was comparable. The K12 expression in the explant culture was the highest but the absolute level was extremely low (Fig. 3A). Expression of Ki67 was the highest in the ColA cultures and α-SMA in the explant culture (Fig. 3A).

We next investigated the amount of $p63\alpha^{br}$ cells in each culture system. A representative level of p63α expression is shown in Fig. 3B. The ColA culture produced the highest percentage of $p63\alpha^{br}$ cells ($p < 0.05$; Fig. 3C). However, the absolute number of $p63\alpha^{br}$ cells was similar in ColA ($8.6 \pm 0.3 \times 10^4$) and explant cultures ($8.1 \pm 0.2 \times 10^4$, $p = 0.28$;

Supplementary Fig. S3A and B) because the cell expansion rate in the explant culture was greater than that in the ColA cultures.

The percentage of $K14^+/K12^-$ cells was similar in all three cultures ($p > 0.05$; Fig. 3D). Interestingly, the lowest percentage of $K14^-/K12^+$ cells was found in ColA cultures ($p < 0.05$; Fig. 3D). Both the ColA and explant cultures contained a higher absolute number of $K14^+/K12^-$ cells ($3.3 \pm 0.1 \times 10^5$ and $7.9 \pm 0.6 \times 10^5$, respectively) than the dispase cultures ($9 \pm 2 \times 10^4$, $p < 0.05$; Supplementary Fig. S3C and D). Explant cultures contained the highest absolute number of $K14^-/K12^+$ cells ($1.16 \pm 0.07 \times 10^5$, $p < 0.05$; Supplementary Fig. S3C and D).

Double staining for K14/Vim in the outgrowth of the explant cultures showed that $K14^-/Vim^+$ stromal cells also migrated with the limbal epithelial cells from the explant; however, $K14^+/Vim^-$epithelial cells were accounted for 69.4% of the outgrowth whereas $K14^-/Vim^+$ stromal cells was only accounted for 7.9% ($p < 0.05$; Fig. 3E and F). In ColA culture, the $K14^-/Vim^+$ cells were still present at the end of culture (Fig. 3E and F).

3.4. Effect of junction disruption on the LSC ex vivo expansion

After isolation of the limbal epithelium, half of the cells were further treated with trypsin to obtain a single-cell suspension, while the other half remained in sheets/clusters. Single cells and cell sheets/clusters were subsequently cultured in the presence and absence of 3T3 cells.

In the presence of 3T3 cells, cultures derived from dispase clusters displayed a more differentiated phenotype in the center of the cluster growth area given by the presence of a higher number of K12 positive cells than the dispase single-cell cultures (Fig. 4A and B) which was confirmed by a higher K12 expression by qRT-PCR (Fig. 4E). The expression level of the putative stem cell markers and Ki67 was similar when grown as single cell suspension and cell cluster in both dispase and ColA cultures (Fig. 4E and F). When analyzing the absolute number of $p63\alpha^{br}$ cells from each culture system, we found a higher number of $p63\alpha^{br}$ cells in the dispase cluster culture ($4.1 \pm 0.2 \times 10^4$) than in the dispase single-cell culture ($1.8 \pm 0.3 \times 10^4$, $p = 0.07$), although the difference was not significant. A comparable number of $p63\alpha^{br}$ cells were generated in both ColA single-cell cultures ($8.6 \pm 0.3 \times 10^4$) and ColA cluster cultures ($9.6 \pm 0.3 \times 10^4$, $p = 0.16$; Fig. 4G).

In the absence of 3T3 cells, no colonies were observed in single-cell cultures. Both dispase and ColA cluster cultures generated LSC growth (Fig. 5A and B). The ColA cluster culture had higher expression levels of the putative stem cell markers and a lower expression level of K12, although these expression levels were not significantly different from those of the dispase cluster culture (Fig. 5C). The ColA cultures generated a greater absolute mean number of $p63\alpha^{br}$ cells ($1.36 \pm 0.06 \times 10^5$) than dispase cultures ($7.7 \pm 0.4 \times 10^4$, $p = 0.02$; Fig. 5D and E), further confirming that the presence of stromal cells in ColA clusters helps maintaining the stem/progenitor phenotype.

4. Discussion

LSCs have been conventionally expanded on growth-arrested murine 3T3 fibroblasts using FBS (Pellegrini et al., 1997). To eliminate cross-contamination from animal products different approaches have been employed in order to establish xenobiotic-free conditions including the use of human serum (Basu et al., 2012; Shahdadfar et al., 2012) and human feeder cells (Chen et al., 2007; Notara et al., 2007; Sharma et al., 2012; Xie et al., 2012; Zhang et al., 2010).

Many studies of LSC isolation have been done to increase the efficacy of the *ex vivo* expansion and to maintain these cells in an undifferentiated state. Some studies have compared the capability of the trypsin with that of dispase or a combination of both to isolate LSCs (Kim et al., 2006; Meyer-Blazejewska et al., 2010). In other studies the efficiency of the LSC expansion using single LSCs isolated by dispase digestion has been compared with that of explant culture (Kim et al., 2004; Koizumi et al., 2002; Zhang et al., 2005). However, there is no clear evidence of which culture method is able to maintain a better stem/progenitor phenotype. In the present report, we addressed this issue by directly comparing the current standard of isolation and culture (the isolation and culture method with dispase) with isolation by ColA and tissue explant culture. The inclusion of the ColA isolation and explant culture in this study permitted the investigation of the role of limbal stromal cells in culture. Moreover, the use of native niche feeder cells would be the best way to culture and maintain the LSC phenotype and to introduce xenobiotic-free conditions in the culture system in a simple manner.

The ColA 37 °C ON INC treatment did not favor the *ex vivo* expansion of LSCs on 3T3 cells, despite providing a significantly higher yield of limbal stromal cells compared to other ColA treatments (Fig. 1). LSCs and stromal cells may compete for the space in the presence of 3T3 cells. It is known that mesenchymal cells have the capacity to quickly attach to plastic dishes (McGrail et al., 2012). Thus, when single cells are seeded, stromal cells might attach first to the dish and saturate the growth space. However, when cell clusters are used, stromal cells and LSCs aggregate, and this aggregation may aid the attachment of LSCs to the culture dish. In addition, ColA may have a toxic, growth-inhibitory effect on LSCs during a prolonged period of incubation (Mohsen et al., 2012). This hypothesis is supported by a lower total number of $K14^{+}/Vim^{-}$ cells isolated by this treatment. Another possibility is that the 3T3 cells and stromal cells may compete to act as feeder cells. It has been shown that when feeder cells are derived from a mixed cell type, different signals may be sent to the cultured cells. Therefore, the combination of signals from different feeder cells could either have a synergistic (Choi et al., 2012) or counteractive effect (Hermann et al., 2006). Growth of limbal epithelial progenitor cells might be inhibited by different signaling from the native stromal cells and the 3T3 cells.

When the efficiency of the epithelial cell isolation by the dispase and ColA methods were compared, accordingly with previous findings (Chen et al., 2011), we observed that ColA digestion is more efficient in the isolation of basal limbal epithelial cells that are embedded in the deep limbal crypts. In addition, ColA digestion could isolate the adjacent limbal stromal cells that are likely components of the LSC niche. Dispase could not efficiently isolate those limbal epithelial cells embedded deep in the limbal crypts. Dispase's cleavage plane is at the level of the lamina densa of the basement membrane (Espana et al., 2003; Gipson and Grill, 1982), whereas ColA digests the connective tissue components and then it removes the deeper stromal collagens (type I, III, and V) but not the basement membrane collagens (Shi et al., 2010). When dispase, ColA and explant cultures were compared in parallel, both the ColA and explant cultures maintained the stem/progenitor phenotype more efficiently. Results from previous studies indicated that LSCs might not migrate from the explant tissue (Koizumi et al., 2002; Tseng et al., 2010; Zhang et al., 2005) and they might undergo intrastromal invasion and epithelial—mesenchymal transition that ultimately implies the loss of LSCs (Tan et al., 2011). We found that the outgrowth of LSCs from the explant is very efficient and consistent. The number of $p63\alpha^{br}$ cells grown from the explant culture was sufficient for successful transplantation (Rama et al., 2010). In addition, explant culture has other advantages over the other two culture systems. Explant culture is simple to prepare, and there is little risk of damaging the LSCs with enzymatic treatment. Moreover, the native niche of the LSCs, meaning native niche cells and factors provided by the tissue, is not disrupted during culture and therefore it might better maintain the phenotype of LSCs

in vitro. Indeed, LSCs transplanted from the explant culture could successfully restore the ocular surface and improve vision in patients with LSCD (Basu et al., 2012). Moreover, the natural corneal stroma is an optimal substrate for the LSC expansion and for the construction of a native-like corneal equivalent (Lin et al., 2012).

When LSC cultures derived from single cells and clusters were compared, we observed that dispase cluster cultures in the presence of 3T3 cells contain a greater number of $p63\alpha^{br}$ stem/progenitor cells. However, there was no difference in the phenotype of the cultured cells derived from ColA single-cells and clusters. It has been proposed that trypsin can damage fragile stem cells (Kim et al., 2006) and that interactions among LSCs can help the *ex vivo* expansion and maintain the stemness in culture (Kawakita et al., 2009). Moreover, the presence of stromal cells might increase the survival of LSCs in the ColA culture. In the absence of 3T3 cells, only dispase and ColA clusters produced LSC growth, showing again the importance of cell junction maintenance between LSC—LSC and LSC—stromal cells in culture.

5. Conclusions

Collectively, our results suggest that the ColA and explant cultures are more efficient than the current standard dispase single-cell culture. Limbal stromal cells from the native niche in both culture systems appear to help in maintaining the stem/progenitor phenotype in culture. Whether these stromal cells in the ColA and explant cultures could continue to serve as LSC niche cells upon transplantation onto the ocular surface and how these stromal cells might influence transplantation outcomes need to be further investigated. In conclusion, the presence of stromal cells might help to maintain the LSCs in an undifferentiated state *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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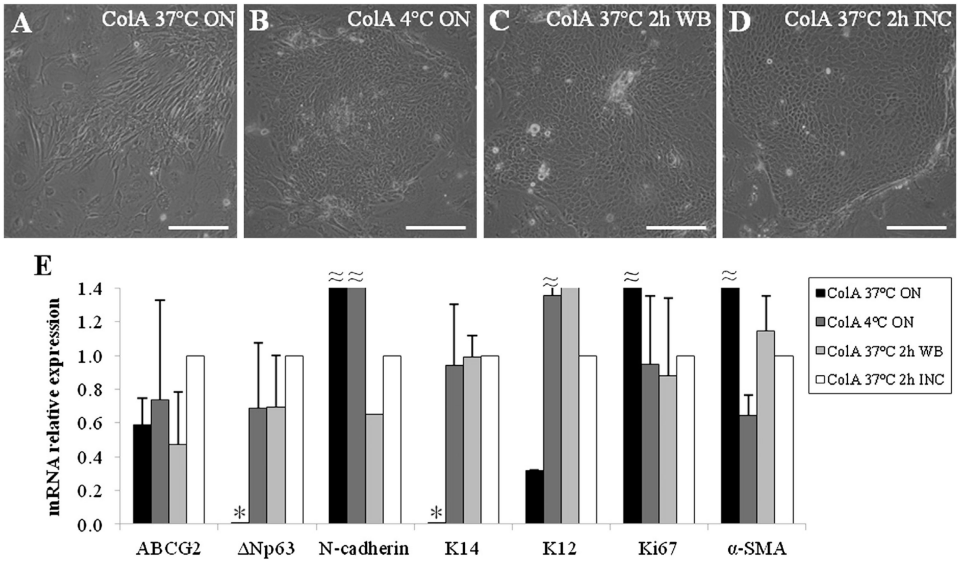

Fig. 1.

Phenotype of cultured LSCs from different ColA treatments. **A**–**D**. Cell morphology after 14 days in culture. **E**. Relative mRNA expression of putative LSC markers, K12, Ki67 and α-SMA (*$p < 0.05$ with regards to ColA 37 °C 2 h INC treatment). Scale bar = 100 μm.

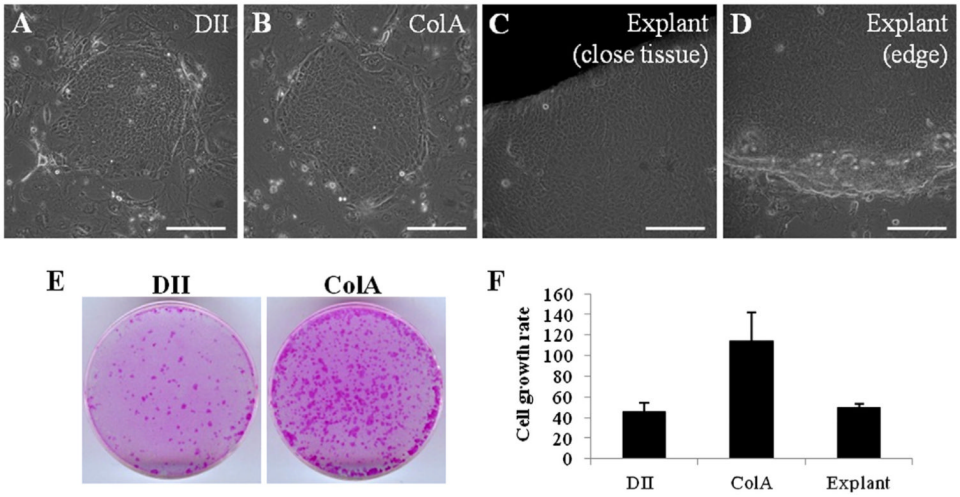

Fig. 2.

Phenotype of cultured LSCs derived from the DII, ColA and explant cultures. **A**–**D**. Cell morphology after 14 days in culture. **E**. Rhodamine B staining of LSCs colonies from DII and ColA cultures. **F**. Cell growth rate of cells from DII, ColA and explant cultures. Scale bar = 10 0 μm. DII: dispase II.

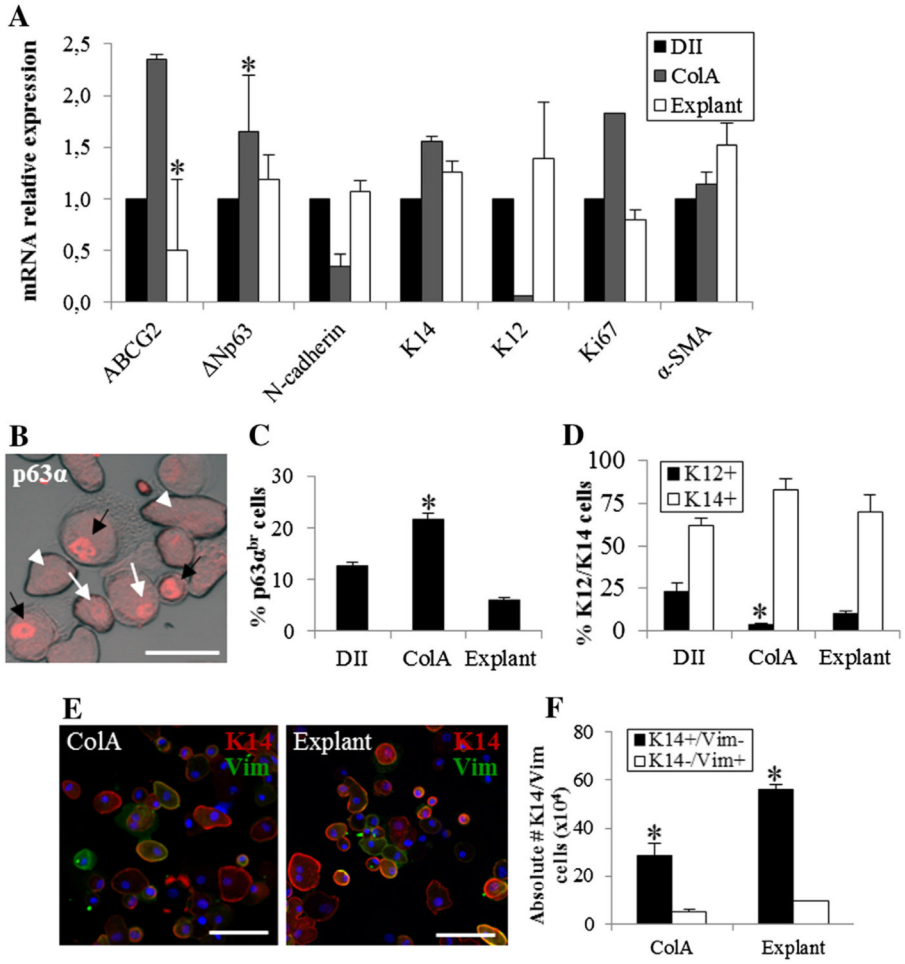

Fig. 3.

Expression profiles of cultured LSCs from the DII, ColA and explant cultures. **A**. Relative mRNA expression of putative LSC markers, K12, Ki67 and α-SMA (*$p < 0.05$ with regards to DII). **B**. Representative expression level of p63α in cultured LSCs; black arrow: $p63α^{br}$ cells; white arrow: p63α weak cells; white arrowhead: p63α negative cells. **C**. Percentage of $p63α^{br}$ cells (*$p < 0.05$). **D**. Percentage of $K12^+$ and $K14^+$ cells (*$p < 0.05$). **E**. Double immunostaining of K14 and Vim. **F**. Absolute cell number of $K14^+/Vim^-$ and $K14^-/Vim^+$ cells on the ColA and explant cultures. *$p < 0.05$. Scale bar = 100 μm. Scale bar = 100 μm.

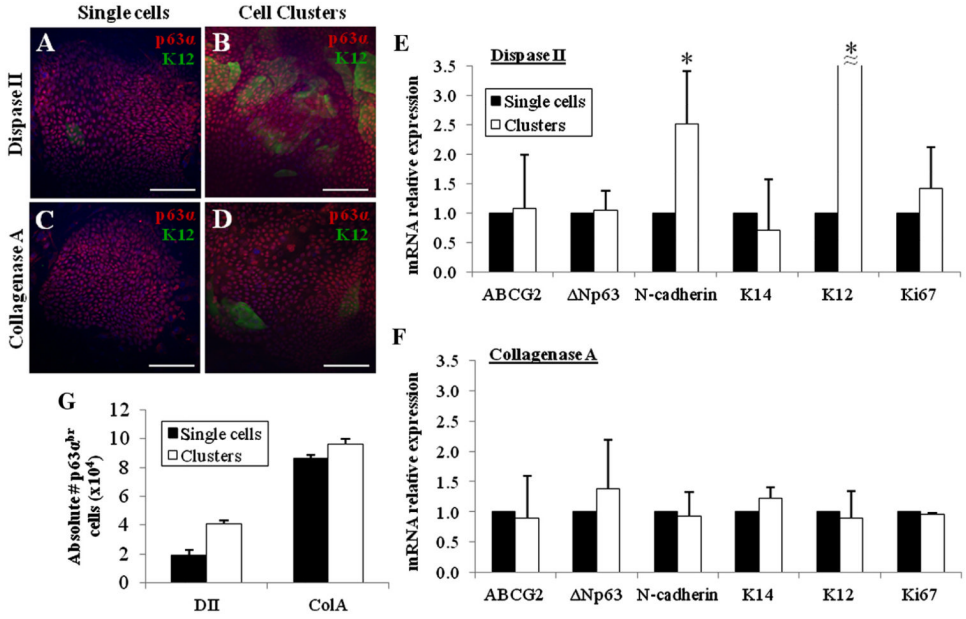

Fig. 4.

Cultured LSCs derived from single cells and cell clusters of DII and ColA cultures in the presence of 3T3 cells. **A**–**D**. Immunocytochemical analysis of p63α and K12 in LSCs. **E**. Relative mRNA expression of markers from DII cultures (**p* < 0.05 with regards to cultured LSCs from single cells). **F**. Relative mRNA expression of markers from ColA cultures. **G**. Absolute cell number of $p63\alpha^{br}$ cells from both DII and ColA cultures. Scale bar = 10 0 μm.

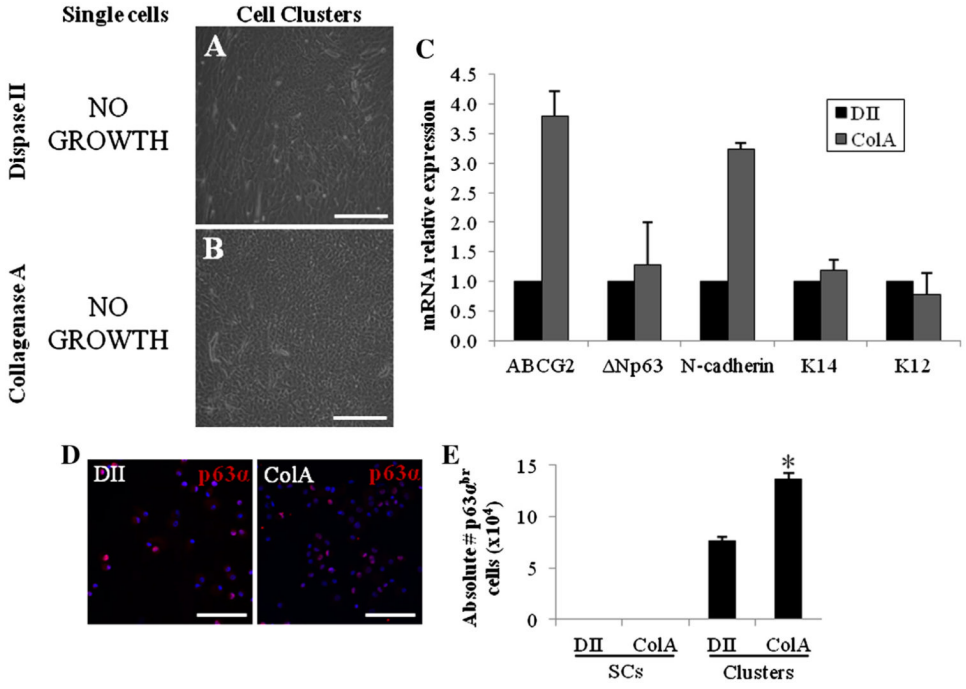

Fig. 5.

Cultured LSCs derived from DII and ColA cell cluster cultures in the absence of 3T3 cells. **A**–**B**. Cluster growth area from DII and ColA cluster cultures. **C**. Relative mRNA expression of markers from DII and ColA cluster cultures. **D.** Immunostaining of p63α. **E**. Absolute cell number of $p63\alpha^{br}$ cells (*$p < 0.05$). Scale bar = 10 0 μm.

Table 1

K14 and Vim expression on freshly isolated limbal epithelial cells after dispase and ColA incubation.

Absolute cell# (%)	DII	ColA 37 °C 2 h INC
$K14^+/Vim^-$	$2.1 \pm 0.2 \times 10^5$ (70.5%)	$3.12 \pm 0.04 \times 10^5$ (86.2%)*
$K14^-/Vim^+$	$6.0 \pm 1.9 \times 10^3$ (2%)	$3.5 \pm 0.5 \times 10^4$ (9.7%)*

Abbreviations: DII: dispase II; ColA: collagenase A; INC: incubator K14, cytokeratin 14; Vim, vimentin.

* $p < 0.05$.