Optimal Isolation and Xeno-Free Culture Conditions for Limbal Stem Cell Function

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PURPOSE. To preserve limbal stem cell (LSC) function in vitro with xenobiotic-free culture conditions.

METHODS. Limbal epithelial cells were isolated from 139 donors using 15 variations of three dissociation solutions. All culture conditions were compared to the baseline condition of murine 3T3-J3 feeders with xenobiotic (Xeno) keratinocyte growth medium at 20% O₂. Five Xeno and Xeno-free media with increasing concentrations of calcium and epidermal growth factor (EGF) were evaluated at 5%, 14%, and 20% O₂. Human MRC-5, dermal (fetal, neonatal, or adult), and limbal stromal fibroblasts were compared. Statistical analysis was performed on the number of maximum serial weekly passages, percentage of aborted colonies, colony-forming efficiency (CFE), p63^α^{bright} cells, and RT-PCR ratio of p63^α/K12. Immunocytochemistry and RT-PCR for p63^α, ABCG2, Bmi1, C/EBP δ , K12, and MUC1 were performed to evaluate phenotype.

RESULTS. Dispase/TrypLE was the isolation method that consistently showed the best yield, viability, and CFE. On 3T3-J2 feeders, Xeno-free medium with calcium 0.1 mM and EGF 10 ng/mL at 20% O₂ supported more passages with equivalent percentage of aborted colonies, p63^α^{bright} cells, and p63^α/K12 RT-PCR ratio compared to baseline Xeno-media. With this Xeno-free medium, MRC-5 feeders showed the best performance, followed by fetal, neonatal, adult HDE, and limbal fibroblasts. MRC-5 feeders supported serial passages with sustained high expression of progenitor cell markers at levels as robust as the baseline condition without significant difference between 20% and 5% O₂.

CONCLUSIONS. The LSC function can be maintained in vitro under appropriate Xeno-free conditions.

Keywords: limbal, stem, cell culture, xeno-free, oxygen

A variety of ocular surface diseases causes limbal stem cell deficiency (LSCD) with painful recurrent epithelial erosions that frequently result in blindness.¹ Recently, limbal transplantation for treatment of LSCD used in vitro expanded limbal or oral epithelial cells since the first application by Pellegrini and De Luca.^{2,3} In vitro culture conditions should be optimal such that limbal stem cells (LSCs) retain their function and do not differentiate.³ While there is no single specific marker of LSCs to our knowledge, markers including p63^α, ABCG2, C/EBP δ , as well as the absence of corneal epithelial differentiation markers K3 and K12, can be used collectively to identify LSCs.⁴ An additional challenge is that most culture methods are based on the use of xenobiotic (Xeno) or allogenic products, such as murine feeder cells, bovine serum, or human amniotic membrane. These products carry the risk of transmitted diseases, tumorigenesis, or precipitation of immunologic rejection, as well as biologic variability.^{5,6} To address these issues, we set out to determine the optimal xenobiotic-free (Xeno-free) culture conditions for LSCs.

Due to the limited amount of starting material, the initial dissociation method and the culture method are critical for

expanding stem cells, while preserving their self-renewal and proliferation potential. During cultivation and aging, epithelial stem cells undergo unidirectional clonal evolution.⁷ Clonal analysis of keratinocytes identifies clonogenic cells giving rise to holoclones, meroclones, and paraclones. Holoclones show hallmarks of stem cell colonies, such as small cells with self-renewal ability,⁸ telomerase activity,⁹ and highest proliferative potential.¹⁰ During clonal evolution, holoclones produce meroclone-forming cells with intermediate proliferating potential, which, in turn, produce paraclones made of transient amplifying progenitors with limited proliferative capacity to generate aborted colonies of terminally differentiated cells.³ For successful clinical transplantation, a 1- to 2-mm² limbal biopsy of up to 17,000 limbal epithelial cells (LECs)/mm² containing approximately 150 p63^α positive progenitor cells must be expanded in vitro to 300,000 cells with 3000 p63^α-positive cells.^{3,11–13} This biopsy typically can propagate approximately 11 holoclones, 39 meroclones, and 8 paraclones.¹⁰

Several different culture conditions have been used in clinical trials using variations of six main protocols.² Each of these methods begins with a small epithelial biopsy, but they

FIGURE 1. Strategy for selection of optimum isolation method and Xeno-free culture conditions. LECs were isolated and dissociated into a single cell suspension that was seeded on feeder cells and cultured in parallel under different conditions. The colonies formed were stained bright pink with rhodamine for identification and counting. ICC, immunocytochemistry (number of p63 α ^{bright} cells); K12, cornea differentiation marker cytokeratin 12.

have fundamental differences as to whether the tissue is grown as explant or dissociated single cells, with or without murine 3T3 fibroblasts as feeders, using amniotic membrane or fibrin as a carrier, and possibly air-lifting to promote epithelial stratification.² In this study, we selected the method of Pellegrini and De Luca (dissociated single cell using 3T3 feeders) as our baseline due to the detailed clonal analysis and experimental evaluation of the method,¹⁰ as well as their proven clinically successful results that have been associated with quality control markers, like p63^{bright} cells and percentage of aborted colonies.^{3,14}

In addition to the molecular markers listed above, LSCs can be identified based on their self-renewal properties with serial cultivation or intensive clonal analysis,¹⁰ presence of long telomeres,¹⁵ or slow cell cycling.¹⁶ The colony-forming efficiency (CFE) assay has been used for evaluating LSCs, since it is much faster and more convenient than clonal analysis or serial cultivation. However, clonogenic ability is different than self-renewal potential¹⁰ and CFE was not correlated with success of transplanted cultured limbal grafts.³ In contrast, successful transplantation was correlated with >3% p63^{bright} cells in the transplant.³ Another quality control criterion for grafts is less than 10% of all colonies to be aborted colonies (paraclones), since the number of paraclones is related inversely to the number of holoclones and paraclones are easier to score.³ In this study, we compared different culture methods based on multiple serial passages (as a practical alternative to the gold standard of clonal analysis), on percentage of aborted colonies, and on p63 α expression as p63 α ^{bright} cells, as well as RT-PCR ratio and p63 α /K12 ratio (Fig. 1).

Recently, there were several attempts to replace potentially harmful xenobiotic materials in limbal epithelial cultures by human instead of murine feeders,^{13,17–21} or human instead of bovine serum,^{22–24} or isoproterenol instead of cholera toxin.²⁵ Xeno-free culture systems using either explants^{26–28} or single cells^{21,29} have been reported recently in the context of good manufacturing practice regulations.³⁰ However, all of these studies have based the evaluation of these techniques on CFE and expression of progenitor markers without stringent functional evaluation of LSC preservation in culture by clonal analysis or multiple passages.¹⁰ The culture technique is critical for preventing differentiation of stem cells in vitro, especially since there currently is no specific marker of LSCs. Even if the cultured cells are transplanted after one passage, irreversible differentiation of stem cells to progenitor cells that we cannot differentiate based on relatively nonspecific progenitor markers, like p63 α , may be enough to compromise the long-term success of the transplants.

Oxygen is a well-known regulator of stem cell fate, with lower oxygen tensions usually preventing stem cell differen-

tiation,³¹ but the relatively few studies of oxygen on limbal stem cells have revealed conflicting results (Miyashita H, et al. *IOVS* 2007;50:ARVO E-Abstract 4608).^{32–38} Lower oxygen tensions of 2% to 5% (estimated to be more representative of the LSC niche) have shown either better^{32,37} or worse³⁴ support of CFE and p63 α expression compared to either 14%, the level typically below the tear film, or atmospheric 20% O₂. In this study, we selected 5% and 14% as potential alternatives to typically used 20% O₂ to evaluate for beneficial effects in preservation of LSC during culture.

To our knowledge, there is no report comparing different limbal progenitor culture conditions using Xeno-free system that is based on stem cell assays, like serial passages or clonal analysis, and not on CFE. The only group so far that has performed clonal analysis for stem cells in culture is that of Pellegrini et al.,¹⁰ whose method we used as a baseline. For this study, we also set up automated quantification of immunocytochemistry for p63 α , as the quality control method that was associated with clinical success by the same group.^{3,39} In this work, we also reported a novel Xeno-free medium variation, based on the medium that was used initially for keratinocytes.⁴⁰ The aim of the present study was to identify optimal retention of functional LSCs in vitro under Xeno-free culture conditions.

MATERIALS AND METHODS

This study followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of the University of Pennsylvania, Philadelphia, PA.

Mouse and Human Fibroblasts as Feeders

The 3T3-J2 mouse fibroblasts were kindly provided by Howard Green (Harvard University, Boston, MA) and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% adult bovine serum and 1% penicillin/streptomycin (all from Invitrogen, Carlsbad, CA). The MRC-5 fibroblasts (CCL-171) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM. Human dermal fibroblasts (HDF) from fetal (PH10605F), neonatal (PH10605N), or adult (PH10605A) skin were purchased and maintained with PM116500 medium (all from Genlantis, San Diego, CA). Limbal stromal fibroblasts were isolated from a total of 6 donors (age range, 26–67 years, two male and four female, preserved from 7–14 days in Optisol) and grown as described previously.¹⁸ The youngest of these donors (26-year-old male) showed the highest and most consistent proliferative capacity during multiple passages, and was selected as the primary cell line to be used as limbal fibroblast feeder cell line in these experiments. All feeders were used at passage 6 to 9,

TABLE 1. List of Six Representative Isolation Methods (of 15 Variations) and number of Donor Rims Used in Each Condition

Number	Isolation Method	Donors, <i>N</i>
1	Trypsin 0.05% × 80 min (4 cycles of 20 min)	15
2	Dispase 1.2 IU/mL × 2 h	12
3	Dispase 2.4 IU/mL × 1.5 h and trypsin 0.25% × 10 min	7
4	Dispase 2.4 IU/mL × 1.5 h and trypsin 0.05% × 10 min	7
5	Dispase 2.4 IU/mL × 2 h and trypsin 0.05% × 1 min	10
6	Dispase 2.4 IU/mL × 2 h and TLE × 10 min	7

culture medium was changed three times a week, cultures were passaged upon reaching 70% to 80% confluence, and maintained at 37°C and 20% O₂. All feeders were plated at a density of 2.4×10^4 cells/cm² and mitotically inactivated with 4 µg/mL of mitomycin C (MMC; Sigma-Aldrich, St. Louis, MO) for 2 hours at 37°C before seeding LECs. This concentration of MMC was selected after trials of 1, 4, and 8 µg/mL as the minimum amount needed to arrest cell growth of each type of feeder cells used in this experiments by cell counts 3 days later, similar to prior reports.¹⁷

Human Limbal Epithelial Cell Culture

For the isolation method experiments, 139 research-consented cadaveric human corneoscleral rims were obtained from the Lions Eye Bank of Delaware Valley (Philadelphia, PA) or the Scheie Eye Institute (Philadelphia, PA) after cornea transplantation. Cadaveric tissue was used only for the experiments comparing different isolation methods. For the rest of the experiments, limbal epithelial specimens 1 × 2 mm were obtained during cataract surgery of 29 volunteers without ocular surface disease, after appropriate informed consent was obtained, following explanation and discussion of the nature and possible consequences of the study. Human limbal epithelial cells were isolated as described previously^{12,21,41} (Table 1, Supplementary Table S1). The whole fresh limbal specimen or the limbal rim from cadaveric donors after 8 mm trephination of the central cornea and scraping of the iris root was incubated with the indicated dissociation solution of either 0.25% Trypsin (Invitrogen) alone or Dispase II (Hoffman-La Roche, Indianapolis, IN) 1.2 or 2.4 IU at 37°C agitated with an orbital shaker at 0.9g for the indicated time. We then peeled the epithelium under a dissecting microscope, incubated in the indicated 0.25% or 0.05% Trypsin/EDTA or TrypLE (Invitrogen), and filtered through a 70-µm cell strainer (BD Falcon; BD Biosciences, San Jose, CA) to dissociate into single cells. All dissociation media, as well as media at first plating after isolation or passage, contained 10 µL/mL Rho Inhibitor Y27632 (Calbiochem; Merck, Darmstadt, Germany), after our preliminary trials of 0, 1, and 10 µL/mL showed improved CFE by

approximately 35% and 15% with addition of 10 and 1 µL/mL Rho inhibitor, respectively, which were confirmed with recent reports from another group.⁴² Cells were collected by centrifugation, suspended in culture media, counted under the microscope with trypan blue (Invitrogen), and seeded at a density of 1.5×10^4 cells/cm² on feeder layer-containing wells. Cell cultures were incubated at 37°C and 95% humidity under 20%, 14%, or 5% O₂ with different culture media (Table 2, Supplementary Tables 2, 3).

Passages, CFE, Holoclone-Forming Efficiency (HFE), and Percentage of Aborted Colonies

Cultures for passages were seeded in parallel at 10,000 cells per well of 6-well plates and passaged weekly before confluent at density 1:3 after dissociation with Trypsin 0.25% containing 10 µL/mL Y27632 (Calbiochem; Merck) which also was added in the initial culture medium. Cell counts and cell diameter analysis were obtained during passages with Scepter 2.0 Automated Cell Counter with a 40-µm sensor (EMD Millipore, Billerica, MA). Cell size was analyzed as cells with diameter up to 10 µm (≤ 10 µm) or larger than 10 µm, based on prior reports of higher expression of p63α in cells smaller than 10 µm.³⁹ For the CFE assay, 500 to 2000 human limbal epithelial cells were plated into 10-cm plates containing growth-arrested 3T3 feeders. Medium was changed on alternate days. Colonies were fixed on day 14 using 4% paraformaldehyde (PFA) for 5 minutes at room temperature, washed with PBS, stained with 2% rhodamine B (Sigma-Aldrich), photographed with a light box, and analyzed with ImageJ software (available in the public domain at <http://rsbweb.nih.gov/ij/>). Colonies with appearance of holoclones, meroclones, and aborted colonies were identified as defined by Green et al.⁷ Holoclones were large with area of at least 10 mm² and smooth perimeter; aborted colonies were smaller than 5 mm², containing mostly large and flattened epithelial cells, and an irregular perimeter; while meroclones were the rest of the colonies typically with a wrinkled perimeter. The CFE and HFE were calculated as percentage of seeded epithelial cells that formed any colonies or holoclones, respectively. The percentage of aborted colonies was calculated as number of aborted colonies/total number of colonies × 100.

Immunocytochemistry and Quantification of p63α^{bright} Cells

Cytospins of dissociated cells and cells cultured on coverslips were used for immunocytochemistry after fixation with 4% PFA for 5 minutes, permeabilization with 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 3 × 5 minutes, followed by PBS wash and blocking with 1% BSA (Sigma-Aldrich) in PBS for 1 hour. Primary antibodies (Supplementary Table S4) for p63α (Cell Signaling Technology, Beverly, MA), p63 (4A4 Abcam, Cambridge, UK), ABCG2 (EMD Millipore), and C/EBPδ (Santa Cruz Biotechnology, Inc., Dallas, TX), cytokeratin 15 (Covance,

TABLE 2. List of Five Basic Culture Media (of the 13 Variations) and Abbreviations

Culture Medium Abbreviation	Xeno-Free	Calcium, mM	EGF, ng/mL	Base Medium	Previously Published
X-KGM	No	1.3	10	DMEM-F12 2:1	Yes ¹⁰
X-SHEM	No	1.05	10	DMEM-F12 1:1	Yes ⁵²
X-MCBD	No	0.03	10	MCDB151	Yes ¹²
XF-SHEM	Yes	1.05	10	DMEM-F12 1:1	No
XF-Ca0.1	Yes	0.1	10	DMEMnoCa-F12 2:1	No

Detailed formulations in Supplementary Table S3. X, Xeno media; XF, Xeno-Free media; KGM, keratinocyte growth medium; SHEM, supplemented hormonal epithelial medium.

FIGURE 2. Selection of isolation method based on yield and CFE. Results of six selected isolation methods (Table 1) of the total of 15 variations are shown, three of them (numbers 1–3) reported previously and three variations of method number 3 (numbers 4–6). They were evaluated for yield as total number of cells isolated from a cadaveric donor corneoscleral rim (A) and for CFE on 3T3-J2 feeders (B). Each bar represents mean and SEM from 6 to 15 different limbal specimens derived from a total of 139 donors. Statistically significant difference of *** $P=0.0001$ or **** $P=0.00001$ with ANOVA/Bonferroni tests between methods 5, 6, and methods 1 to 3 in (A), and methods 1 to 4 in (B). Method 6 (Dispase 2.4 IU/mL for 2 hours and TLE for 10 minutes) consistently showed the best results.

Princeton, NJ), cytokeratin 12 (Santa Cruz Biotechnology, Inc.), and mucin 1 (Santa Cruz Biotechnology, Inc.) at 1:100 dilution were applied for 1 hour, washed with blocking solution, and incubated with their respective donkey-raised secondary antibody Alexa Fluor 488 or 555 (Molecular Probes, Eugene, OR) at 1:1000 dilution for 1 hour, washed with PBS, and counterstained with ProLong Gold with DAPI (Molecular Probes), with all incubations at room temperature. Negative controls were either omitted primary or isotype control antibody.

Fluorescence images were acquired with an Olympus IX81 microscope (Olympus, Tokyo, Japan) running Metamorph 7 software. Images for quantification were from cytopins, and had standardized exposure times for 4',6-dimidino-2-phenylindole (DAPI) and p63 α signals. Image analysis was performed with ImageJ software, by selecting nucleus counter on the DAPI image, and region of interest (ROI) analysis for acquiring fluorescence intensity on DAPI and p63 α images. A minimum of 500 cells was counted for each specimen. Data were exported to Excel, where the ratio of p63 α /DAPI intensity was calculated and the distribution of intensity was graphed. In initial experiments, the cell size from bright field images was correlated with p63 α , as described by Di Iorio et al.,³⁹ with a yield of high intensity p63 α cells between 4% and 9% (5.8 ± 2.5 , $n=6$) for the baseline culture condition, which correlated with intensity ratio of p63 α /DAPI of 1.5 or higher, and that ratio was selected as threshold. The percentage of p63 α ^{bright} cells was multiplied with the total number of cells in each plate to yield absolute number of p63 α ^{bright} cells.

RNA Extraction, Real-Time RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and RNeasy Mini and QIA Shredder (Qiagen, Venlo, The Netherlands) according to manufacturer's protocol. RNA quality and quantity were measured using Nanodrop 100 (Thermo-Fisher Scientific, Waltham, MA) and Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA). The high capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA) was used to prepare cDNA via reverse transcription. Relative mRNA expression was assessed using an ABI prism 7900 HT sequence detection instrument (Applied Biosystems). Primers for p63 α (Hs00978338_m1), ABCG2 (Hs01053796_m1), C/EBP δ (Hs00270931_m1), Bmi1 (Hs00180411_m1), and K12 (Hs01057907) were obtained from Applied Biosystems. Human endogenous control arrays (4366071; Applied Biosystems) were used to select the most stable endogenous control genes for these experiments. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905_m1), peptidylpropyl Isomerase A (PPIA, Hs99999904_m1), and Ubiquitin C (UBC, Hs00824723_m1) were selected, obtained from Applied Biosystems, and used as housekeeping internal control genes. No-template controls were run for each assay to ascertain lack of contamination. Comparative threshold cycle (C_T) method was used to measure relative change in gene expression. All samples were run in four replicates and the final results were an average of at least four experiments.

Statistical Analysis

Statistical analysis of results were carried out using SAS 9.2 software (GraphPad Software, Inc., La Jolla, CA). One-way ANOVA with Bonferroni post hoc tests was used, as well as Spearman correlations. All error bars represent SEM values. Sets of data producing $P < 0.05$ were considered statistically significant.

RESULTS

Combined Dispase and TrypLE Single Cell Dissociation Provided Best Yield, Viability, and CFE

Corneoscleral rims from a total of 139 cadaveric donors, 78 male and 61 female of age 58 ± 12 years (range, 18–75), were stored in Optisol for an average of 13 ± 6 days (range, 3–35) before isolation, and were used for evaluation of a total of 15 variations of dissociation methods (Supplementary Table S1), with six representative variations (three previously published) shown in Table 1. All methods were evaluated for yield as number of cells isolated per whole cornea rim, viability with trypan blue, and CFE (Supplementary Fig. S1). Methods that had been reported previously, namely trypsin 0.05% for 80 minutes,⁴¹ Dispase 1.2 IU/mL for 2 hours,²¹ and Dispase 2.4 IU/mL for 1.5 hour, followed by scraping and trypsin 0.25% for 10 minutes,¹² numbered as method 1, 2, and 3, respectively (Fig. 2), were tried first, but in our hands they consistently produced low yield and minimal CFE, with method 3 being the best among the three. We then tried 12 variations of method 3, adjusting the concentration and exposure time of Dispase and trypsin/TrypLE (Supplementary Table S1, Supplementary Fig. S1), and the three best performing methods are shown in Figure 2 as methods 4 to 6.

Statistical analysis of all isolation methods showed that yield with isolation methods 1 to 3 was significantly lower than several other methods, including methods 5 and 6 ($P=0.0001$, Fig. 2, Supplementary Fig. S1). No significant difference was

FIGURE 3. Evaluation of Xeno-Free media for growth of LECs. X-KGM medium was selected as the baseline medium, included in every experiment, and dissociated LECs (method 6) were seeded at the same density and grown in parallel. XF-Ca0.1 medium supported more weekly passages to senescence than any other medium (A), while it showed low percentage of aborted colonies (B), and not significantly different CFE and HFE (C) compared to the baseline medium. Low calcium (0.1 mM) Xeno-free medium supported more passages than higher calcium media, all with EGF 10 ng/mL (D). The XF-Ca0.1 media supported more passages when supplemented with 10 to 20 ng/mL EGF (E). Immunocytochemistry for p63 α^{bright} cells, quantified and normalized for baseline medium X-KGM, showed significantly higher expression in low calcium Xeno-free medium and lower expression in X-MCBD medium (F). The RT-PCR ratio of p63 α /K12 normalized for X-KGM was not significantly different among the different media (G). Each bar represents mean and SEM from 6 to 23 fresh limbal specimens. Differences of *** $P = 0.0001$, ** $P = 0.009$, and * $P = 0.01$ or 0.019 with ANOVA between all media variations in all oxygen tensions (20%, 14%, and 5%), but for simplicity results from only 20% O₂ are shown in this figure. Abbreviations of media names as in Table 2. Xeno-free low calcium medium with EGF 10 ng/mL showed the best performance.

seen for viability among the different methods varying between 72% and 93% ($P = 0.1635$, Supplementary Fig. S1B). The CFE with gentle trypsinization methods 5 and 6 was significantly higher than methods 1 to 3, and several variations of method 3 ($P = 0.00001$, Fig. 2, Supplementary Fig. S1). Multivariate analysis of variables donor age, preservation time, use of Dispase, and use of trypsin showed that yield was affected negatively by preservation time ($P = 0.0004$) and positively by use of trypsin ($P = 0.00001$), viability was negatively affected by use of trypsin ($P = 0.0302$), and CFE was negatively affected by donor age ($P = 0.0008$). Yield was negatively correlated with preservation time ($\rho = -0.52$, $P = 0.00001$) and positively correlated with dispase ($\rho = 0.58$, $P = 0.00001$), and CFE was negatively correlated with preservation time ($\rho = -0.47$, $P = 0.0001$), and positively correlated with yield ($\rho = 0.83$, $P = 0.0011$) and with dispase ($\rho = 0.34$, $P = 0.0084$).

Eventually, isolation method number 6, Dispase 2.4 IU/mL for 2 hours, scraping, and TrypLE for 10 minutes, was selected as the preferred isolation method for its consistently high yield, viability, and CFE in cadaveric tissue isolation experiments. For cadaveric tissue, this isolation method showed a yield of

$1,352,687 \pm 47,814$ cells/whole sclerocorneal donor rim, viability of $90\% \pm 2\%$, and CFE of $0.077\% \pm 0.02\%$ (mean $\pm$ SEM, Fig. 2, Supplementary Fig. S1). This dissociation method was used for all subsequent experiments using fresh, rather than cadaveric, limbal tissue. Subsequent experiments using 29 fresh limbal epithelial specimens, 6 male and 23 female of mean age 70 ± 8 years (range, 25–90) with this isolation method showed yield of $305,857 \pm 59,657$ cells/mm², viability of $92\% \pm 2\%$, CFE of $2\% \pm 0.5\%$, and percentage of aborted colonies $8\% \pm 3\%$ (mean $\pm$ SEM).

Xeno-Free Media and Oxygen Tension With Murine Feeders

Limbal epithelium specimens from 23 patients of mean age 70 ± 11 years (range, 54–90), 5 male and 18 female, were used for isolation of limbal epithelial cells with Dispase 2.4 IU/mL for 2 hours followed by TrypLE for 10 minutes. The cells then were seeded in parallel on 3T3-J2 feeders and cultured with different media (see Table 2 for list of most important media and Supplementary Table S2 for complete list of media) under 20%,

FIGURE 4. Effect of oxygen tension on LEC culture. Xeno-free medium with calcium 0.1 mM and EGF 10 ng/mL supported more weekly passages of LECs to senescence under 20% O₂, while X-KGM performed better under 5% O₂ (A). Immunocytochemistry for p63α^{bright} quantified and normalized for baseline medium X-KGM at 20% O₂ showed higher expression in the Xeno-free medium under 20% O₂ (B). The RT-PCR ratio of p63α/K12 normalized for X-KGM at 20% O₂ was not significantly different among the different media and O₂ tensions (C). Each bar represents mean and SEM from 6 to 23 fresh limbal specimens. Difference of *** $P = 0.0001$ and * $P = 0.01$ with ANOVA, between all media variations in all oxygen tensions (20%, 14%, and 5%), but for simplicity results from these two media are shown in this figure.

14%, or 5% O₂ (Supplementary Fig. S2). Then, X-KGM medium at 20% O₂ was included in every experiment as the baseline condition. Outcomes were evaluated as the maximum weekly passages until senescence, as well as percentage of aborted colonies, CFE, and HFE at initial seeding. A maximum of 10 weekly serial passages was noted under certain conditions. The XF-Ca0.1 medium supported more passages, while X-SHEM, X-MCBD, and XF-SHEM supported fewer passages than the baseline X-KGM medium ($P = 0.0001$, Fig. 3A, Supplementary Figs. S2A, S2B). Multivariate analysis of effect of donor age, sex, media, and oxygen tension as variables on the number of passages showed significant effect of donor age ($P = 0.002$) and culture media ($P = 0.0001$), but no effect of sex ($P = 0.061$) or oxygen ($P = 0.434$).

The percentage of aborted colonies was higher for higher calcium and for X-MCBD media ($P = 0.019$, Fig. 3B, Supplementary Figs. S2G, S2H), while the rest of the media were not significantly different from the baseline value of 8%. The CFE was higher for X-SHEM and XF-SHEM media under 14% O₂ only ($P = 0.003$, Fig. 3C, Supplementary Figs. S2C, S2D), while HFE was higher for X-SHEM at 14% O₂, and XF-SHEM at 20% and 14% O₂ ($P = 0.009$, Fig. 3C, Supplementary Figs. S2E, S2F). Low (0.1 mM) calcium Xeno-free medium at 20% O₂ supported more passages than the higher concentrations of 0.4, 1.05, and 1.3 mM when epidermal growth factor (EGF) was 10 ng/mL ($P = 0.0001$, Fig. 3D, Supplementary Fig. S2A), and had low percentage of aborted colonies (Fig. 3B,

FIGURE 5. Comparison of human feeders with Xeno and Xeno-free media. Human lung fibroblasts MRC-5 and HDF, either F-HDF, N-HDF, or A-HDF feeders, supported LEC cultures with low percentage of aborted colonies (A), high expression of p63α^{bright} cells by immunocytochemistry (B), and high RT-PCR ratio of normalized p63α/K12 expression (C), with X-KGM and with low calcium (0.1 mM) Xeno-free (XF) medium with EGF 10 ng/mL. Each bar represents mean and SEM from 6 to 14 fresh limbal specimens, normalized to the baseline condition of mouse 3T3-J2 feeders with X-KGM medium. Significant differences of *** $P = 0.0001$ for aborted colonies, * $P = 0.022$ for ICC and * $P = 0.03$ for RT-PCR with ANOVA tests. The MRC-5 and F-HDF human feeders were selected for further evaluation.

Supplementary Fig. S2G). The EGF concentrations of 10 and 20 ng/mL supported more passages among concentrations from 0 to 50 ng/mL in low calcium Xeno-free (XF-Ca0.1) medium ($P = 0.0001$, Fig. 3E, Supplementary Fig. S2B). The number of small cells ($\leq 10 \mu\text{m}$) during passages showed no statistically significant difference among different conditions, but conditions with less passages tended to show lower numbers of small cells (Supplementary Fig. S4, results from baseline condition shown). We observed that conditions that showed early large colonies tended to have larger, flatter, and more differentiated cells, and were exhausted in culture sooner than conditions with relatively smaller, but compact colonies of small round cells, and clonal analysis on more than 30 clones

FIGURE 6. Further evaluation of human fibroblast feeders MRC-5 and F-HDF with Xeno-free medium. Human lung fibroblasts MRC-5 and F-HDF feeders supported LEC cultures of different morphology at first passage; colonies looked more round and separated from the 3T3-J2 or MRC-5 feeders with either Xeno or Xeno-free medium, while F-HDF feeders supported colonies that were more difficult to identify among the feeders ($\times 5$, tissue culture phase microscope, [A] first column). In higher magnification ($\times 20$), phase photographs and immunocytochemistry for progenitor cell marker p63 α (red), with nuclear counterstain DAPI (blue) overlay ([A], right column) showed presence of limbal progenitor cells growing in more (3T3-J2 or MRC-5) or less (F-HDF) distinct colonies ([A], right column). The RT-PCR ratio of p63 α /K12 showed that MRC-5 feeders with XF medium could support favorable ratio for progenitor cell expansion comparable to 3T3-J2 feeders with X-KGM medium for multiple weekly passages (B). Points represent mean and SEM of four replicates, normalized to isolated limbal epithelial cells before seeding (P0) in parallel in the four different conditions under 20% O₂. Xeno-free (XF) medium had calcium 0.1 mM and EGF 10 ng/mL.

showed that the size and morphology of the clone could not predict whether it would eventually be holoclone or meroclone.

Immunocytochemistry for limbal progenitor marker p63 α^{bright} cells quantified at the first passage showed more p63 α^{bright} cells in cultures grown with low calcium Xeno-free media with 10 ng/mL EGF and less cells in X-MCBD medium ($P = 0.01$, Fig. 3F, Supplementary Fig. S3A), but no significant difference among the rest of the media and the baseline condition, which had an average number of $8\% \pm 2.3\%$ of

p63 α^{bright} cells cultured with X-KGM. Immunocytochemistry expression for limbal stem cell markers was high for p63 α , ABCG2, C/EBP δ , and K15, and low or absent for K12 and MUC1 at the first passage for all conditions evaluated. We found it difficult to predict the amount of passages from evaluating LSC marker expression under the microscope, except in extreme cases of only 1 to 2 passages in which we could see more K12 expression and less p63 α^{bright} cells at P1. The quantified p63 α^{bright} expression in cytopins showed a positive correlation with the number of passages that was

close, but not statistically significant (ρ 0.34, P = 0.063). The RT-PCR ratio of p63 α /K12 differentiation marker expression showed no significant difference among all the media used (P = 0.79, Fig. 3G, Supplementary Fig. S3B).

Oxygen tension of 20% supported more passages with low calcium Xeno-free medium, while 5% O₂ supported more passages with baseline X-KGM media (P = 0.0001, Fig. 4A). Xeno-free medium under 20% O₂ also showed more p63 α ^{bright} cells with immunocytochemistry (P = 0.01, Fig. 4B), and no significant difference in RT-PCR ratio p63 α /K12 (Fig. 4C) or aborted colonies (Fig. S2G). Univariate analysis on p63 α ^{bright} cells showed higher expression in 20% than 14% or 5% O₂ (P = 0.031), while there was no significant effect of oxygen on RT-PCR ratio of p63 α /K12 (P = 0.12).

Xeno-free medium with low calcium 0.1 mM and EGF 10 ng/mL was selected as the best performing Xeno-free medium to be evaluated with human feeders under 20% and 5% oxygen tension.

Human Feeders With Xeno-Free Medium

Five different human fibroblast feeders, namely lung fibroblasts MRC-5; dermal fibroblasts, either fetal (F-HDF), neonatal (N-HDF), adult (A-HDF); and adult limbal fibroblasts were tested as alternatives to murine fibroblast 3T3-J2 feeders. Limbal epithelial specimens from 14 human volunteers of age 66 $\pm$ 4 years (range, 25–87), 2 male and 12 female, were dissociated into single cell suspension, and seeded in parallel onto different feeders with either X-KGM or Xeno-free medium with 0.1 mM calcium and 10 ng/mL EGF, and grown under 20% or 5% O₂. The condition of 3T3-J2 feeders with X-KGM at 20% O₂ was the baseline condition included in every experiment.

The MRC-5, F-HDF, N-HDF, and baseline 3T3-J2 feeders had much lower percentage of aborted colonies than A-HDF feeders (P = 0.0001, Fig. 5A). The MRC-5, F-HDF, and A-HDF feeders with Xeno and Xeno-free media, and N-HDF with Xeno media showed more p63 α ^{bright} cells (P = 0.022, Fig. 5B) and higher RT-PCR ratio p63 α /K12 (P = 0.03, Fig. 5C). The A-HDF could not support more than 3 passages of LECs in culture, while MRC-5, F-HDF, and 3T3-J2 supported at least eight passages, and N-HDF supported approximately five passages. Limbal fibroblasts could support only minimal growth of LECs, with very few and small differentiated colonies that could be only passaged once or twice. Limbal fibroblasts, therefore, were not analyzed further (results not shown). No significant difference was found between 20% and 5% O₂ (P = 0.35). The MRC-5 and F-HDF feeders at 20% O₂ were selected for further analysis.

Colonies grown on MRC-5 feeders with Xeno-free medium at 20% O₂ were compact, round, and separated from the feeders, similar to colonies grown on 3T3-J2 feeders, while colonies grown on F-HDF feeders were more difficult to identify among the feeders (Fig. 6A). Cell size analysis during multiple passages showed no statistically significant difference between MRC-5 or F-HDF feeders with XF media and the baseline condition (Supplementary Fig. S4, results from MRC-5 and baseline condition shown). The CFE showed a gradual decrease during multiple passages that was not statistically different between MRC-5, F-HDF feeders, and baseline condition (Supplementary Fig. S5, results from MRC-5 and baseline condition shown). Colonies on all feeders showed good expression of p63 α at P1 (Fig. 6A), which lasted throughout multiple passages. The RT-PCR ratio of p63 α /K12 over multiple passages showed similar results of sustained high ratio for MRC-5 feeders with Xeno-free medium and for baseline 3T3-J2 feeders with X-KGM, while F-HDF feeders with Xeno-free medium had comparable levels, but with less stable course, and 3T3-J2 with Xeno-free medium dropped the ratio earlier

than with X-KGM medium (Fig. 6B). Colonies grown on MRC-5, F-HDF, and 3T3-J2 feeders with Xeno-free medium demonstrated high expression of p63 α , ABCG2, C/EBP δ , and K15, with minimal or no expression of cornea (K12) and conjunctiva (MUC1) differentiation markers from the first passage (Supplementary Fig. S6, results from MRC-5 feeders shown). Expression of LSC genes with immunocytochemistry persisted to passage 7 and it was more robust in colonies grown on MRC-5 feeders than F-HDF, and similar to expression in colonies grown on 3T3-J2 feeders with Xeno medium (Figs. 7A–D, results from MRC-5 feeders shown). The RT-PCR for p63 α , ABCG2, C/EBP δ , Bmi1, and K12 during multiple passages showed robust upregulation of mostly p63 α and ABCG2 with downregulation of K12, with a pattern similar in colonies grown with MRC-5 or F-HDF or 3T3-J2 feeders, with F-HDF feeders showing a less stable expression pattern (Fig. 7E, results from MRC-5 feeders shown). A summary of all the results obtained is presented in Supplementary Table S5.

Overall, MRC-5 feeders with Xeno-free low calcium medium at 20% O₂ were as robust as the baseline 3T3-J2 feeders with Xeno medium in supporting limbal epithelial stem cell culture. Cells grown in this condition exhibited appropriate colony and cell morphology, sustained expression of appropriate markers with immunocytochemistry and RT-PCR, low percentage of aborted colonies, and the ability to support multiple passages, indicating preservation of LSCs during cultivation.

DISCUSSION

To our knowledge, our study is the first to demonstrate that limbal stem cells can be expanded in vitro under Xeno-free conditions, by sustained multiple passages until exhaustion, low percentage of aborted colonies, and appropriate phenotype with high expression of the limbal progenitor marker p63 α . The efficacy of this Xeno-free system is as robust as the baseline culture condition with xenobiotic murine feeders, bovine serum, and cholera toxin. This baseline condition reported by Pellegrini et al.³ and Rama et al.,¹⁴ based on the Green's lab culture method, has been validated extensively with clonal analysis as well as clinically, using percentage of p63^{bright} cells and aborted colonies. Our results matched these prior reports of this baseline condition and demonstrated that only appropriate Xeno-free culture conditions can support functioning LSCs in vitro for multiple passages with appropriate phenotype.

In our study human lung fibroblasts MRC-5; dermal fibroblasts of fetal, neonatal, or adult origin; as well as limbal stromal fibroblasts were tested in parallel as feeders. Our results agreed with prior publications of MRC-5 feeders used as feeders for epithelial cells,^{21,43,44} but for the first time to our knowledge we demonstrated that these feeders can support multiple passages under Xeno-free conditions, while keeping progenitor cell markers expressed in similar levels with baseline Xeno condition. The MRC-5 is a diploid cell line derived from human fetal lung tissue that can attain 48 population doublings before declining. It is not carcinogenic in animal models and it has been used extensively for the production of human vaccines.^{45,46} The MRC-5 feeders do not depend as much on bovine serum, but in our case they performed well with 5% human serum, which was shown recently to be the best alternative to bovine serum for culturing conjunctiva cells.⁴³ Limbal epithelial cultures on MRC-5 feeders had higher CFE without serum compared to 10% bovine serum.²¹ Human dermal fibroblasts, in our hands, showed significant variability in their ability to support cultures. These results agreed with prior reports using Xeno media, where only 2 of 4 newborn and 1 fetal dermal fibroblast

FIGURE 7. Limbal epithelial cells express appropriate markers of LSCs in multiple passages under Xeno-free culture conditions. Colonies at passage 7 expressed limbal progenitor cell marker p63α or p63 (red, [A–D]) as well as ABCG2 (green, [A]), C/EBPδ (green, [B]), cytokeratin K15 (K15, green, [C]), and cornea differentiation marker cytokeratin 12 (K12, green, [D]), and were counterstained with DAPI (blue, [A–D]) for overlays ([A–D]). The RT-PCR expression (mean and SEM from 4 replicates) of limbal progenitor cell markers p63α, ABCG2, Bmi1, C/EBPδ, and differentiation marker K12, was normalized to isolated limbal epithelial cells before seeding (P0), and was preserved for multiple weekly passages (E).

lines could support growth of LECs,¹³ and human dermal fibroblasts of unknown donor age supported growth of LECs on amniotic membrane.¹⁷ Human limbal stromal fibroblasts have been shown to support limbal epithelial culture,¹⁸ and they would have the advantage of origin next to the LSC niche, and readily availability from cadaveric donor tissue, but in our hands they could not support robust colonies or multiple passages. Our results are in agreement with results from the only other group to our knowledge that tried limbal fibroblasts as feeders, where they could support only one to two passages (Harkin DG, personal communication, 2013).¹⁸ Despite the advantage of limbal fibroblasts' origin next to the native niche, these cells are not specialized niche cells, and the specific culture conditions that have been formulated using different kinds of fibroblasts likely make it difficult to reproduce the limbal niche and support LSC cultures in multiple passages. Overall, MRC-5 feeders showed the most consistent support of LSCs in Xeno-free culture in levels similar to baseline Xeno conditions, when evaluated by multiple passages, percentage of aborted colonies, and expression of limbal progenitor markers.

Oxygen tensions of 20%, 14%, and 5% in this study did not show statistically significant consistent universal effect on supporting limbal cultures. Instead, we observed that different oxygen tensions had varying effects on specific combinations of feeders and media. For example, 5% O₂ supported more passages on 3T3-J2 feeders with Xeno media, while 20% O₂ was better on the same feeders with Xeno-free media. We also did not find any statistically significant difference between 5% and 20% O₂ among cultures using human feeders with Xeno-free media. We suggested that this differential effect of oxygen is responsible for the conflicting results in the literature, where some publications advocate beneficial effect with 2% to 5% O₂,^{32,37} and others with 14% O₂.³⁴ Other studies suggest that hypoxia promotes terminal differentiation of limbal epithelial cells,³³ while hypoxia is a well recognized cause of LSCD.⁴⁷ Of note, our results from an extended number of samples cultured in parallel demonstrated that the effect of oxygen on one combination of feeders and media does not predict that the same effect will be true in another combination of feeders-media, likely because they create a different niche-like environment.

A new formulation of Xeno-free medium, based on the standard keratinocyte growth medium, showed the best performance. We found that low calcium and Xeno-free additives that have been found to be beneficial in other culture systems, like albumin, transferrin, selenium, nonessential amino acids, and pyruvate, allowed us to decrease the amount of human serum to 5%, but 3T3-J2 feeders could not support growth of limbal epithelial cells under serum-free conditions. Our results agreed with the previous report on the effect of lower calcium and EGF on preserving stem cell phenotype.^{12,48} This new medium formulation is a Xeno-free alternative to the baseline Xeno medium, since it performed well with murine 3T3-J2 feeders, but it performed even better with human MRC-5 feeders.

Isolation methods with gentle trypsinization showed improved colony-forming efficiency, likely due to lower toxicity of the trypsin as well as the potential presence of more cell clusters at the beginning of the culture. Donor age, in this study, showed a statistically significant negative effect on number of passages in cultures grown from healthy volunteers and on CFE in cultures grown from cadaveric donor tissue. For cadaveric tissue, prolonged preservation decreased yield and CFE. These results were in agreement with prior reports of negative effect of age on CFE,⁴⁹ while explant cultures showed no effect of age,⁵⁰ but a negative effect of increased preservation time on CFE⁵¹ or a delay on initial expansion of

the culture.⁵⁰ Of note though, a specimen from an older than 90-year-old healthy volunteer, in our study, still grew colonies that sustained six passages. So, younger age probably is beneficial in cases where this option may exist, but older age does not preclude good stem cell function.

Limitations of this study arise from our ability to replicate previously published methods exactly the way they were performed initially. Other limitations include the inherent variability of individual human tissue, both for limbal samples as well as for human feeders, which we tried to mitigate by using multiple samples and growing cultures in parallel, always including the same baseline condition.

In conclusion, our results demonstrated that LSC function can be preserved in vitro under Xeno-free conditions. That allows for the best support of lifelong corneal epithelial regeneration, while minimizing the risk of transmitting any disease through xenobiotic factors. To our knowledge, this is the first extensive comparison of Xeno-free methods that has used rigorous evaluation of stem cell function, namely serial passages, as well as clinically validated quality control measurements of quantification of p63^α^{bright} cells, and percentage of aborted colonies. Our results showed that certain Xeno-free conditions can support limbal epithelial stem cell culture under good manufacturing practices facilitating translation into clinical applications.

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