Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Trustees of the University of Pennsylvania

2. **Reporting Period (start and end date of grant award period):** 1/1/2010-12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Gearline R. Robinson-Hall, BSF

4. **Grant Contact Person's Telephone Number:** 215-746-6821

5. **Grant SAP Number:** 4100050912

6. **Project Number and Title of Research Project:** 13 - Ocular Surface Limbal Stem-Cell Therapy for Corneal Regeneration

7. **Start and End Date of Research Project:** 4/10/2012 – 12/31/2013

8. **Name of Principal Investigator for the Research Project:** John D. Gearhart, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 167,938.23

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Goings, Davida	Tissue Culture Technician	100%	$68,763.17

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Gearhart, John	Principal Investigator	< 1%
Stasi, Kalliopi	Co-Investigator	75%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes___X___ No_____

If yes, please indicate the source and amount of other funds:

NEI/NIH Grant K12 EY015398: The PENN Vision Clinical Scientist Program ($78,369) and Research to Prevent Blindness unrestricted grant awarded to Scheie Eye Institute (without specific amount devoted to this project)

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No_____X_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the

application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
None	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes_____ No____X_____

If yes, please describe your plans:

12. Future of Research Project. What are the future plans for this research project?

We may continue research in this subject when an investigator with appropriate background and interests is involved. Kalliopi Stasi who was an investigator central to this project has moved to the pharmaceutical industry.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female	2			
Unknown				
Total	**2**			

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic	2			
Unknown				
Total	**2**			

	Undergraduate	Masters	Pre-doc	Post-doc
White	2			
Black				
Asian				
Other				
Unknown				
Total	**2**			

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

We set up culture and research in the field of ocular surface limbal stem cells for the first time in the University of Pennsylvania.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___X___ No_____

If yes, please describe the collaborations:

The Lions Eye Bank of Delaware Valley for supply of donor tissue

16(B) Did the research project result in commercial development of any research products?

Yes_____ No____X____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes___X___ No_____

If yes, please describe involvement with community groups that resulted from the research project:

The Lions Eye Bank of Delaware Valley for supply of donor tissue

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a

performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

The aim of this project was to preserve limbal stem cell (LSC) function *in vitro* with xenobiotic-free culture conditions. Limbal stem cells (LSCs) are responsible for the constant renewal of the cornea epithelium that is necessary for clear vision. Damage to LSCs can cause limbal stem cell deficiency (LSCD) with painful light sensitivity and blindness. The only definite treatment for LSCD is restoration of the LSC function by limbal transplantation. Unilateral cases of LSCD may benefit from limbal autograft from the fellow healthy eye of the same patient. The main risk of this procedure is removal of too many LSCs causing LSCD in the healthy eye. In order to minimize this risk, a small area of 1-2mm^2 can be cultured and expanded before transplantation. The success rate of limbal transplantation depends on the amount of LSCs preserved in the transplant. Under usual cell culture conditions, stem cells tend to differentiate and lose their potential for lifelong regeneration. Different research groups worldwide have used different culture conditions in order to maintain LSCs in culture. There is no consensus and no direct head to head comparison of different culture conditions of LSCs for preservation of their regeneration potential.

The objective of this project was to develop and directly compare culture conditions of LSCs for use in future clinical transplantation in patients with LSCD.

<u>Specific Aim 1</u>: Determine the most successful and consistent method of enzymatic or combined enzymatic/mechanical isolation of LSCs from limbal tissue for efficient and successful cultivation, based on yield, viability, colony forming efficiency (CFE) and expression of potential LSC markers. The aim was achieved.

<u>Specific Aim 2</u>: Explore ways to avoid the use of animal products in the feeder supportive cells (mouse fibroblasts) and the media (bovine serum) for successful cultivation of LSCs for future clinical transplantation that is xeno-free, while supporting growth of LSCs measured with CFE, percentage of holoclones, preservation of LSC markers, and subcultivation. The aim was achieved.

<u>Specific Aim 3</u>: Determine optimum oxygen levels for successful culture of LSCs, comparing traditional culture at 20% O_2 versus lower amounts of O_2 (14%, 5% and 2%),

based on CFE, percentage of holoclones, preservation of LSC markers, and ability to subcultivate them. The aim was achieved.

MATERIALS AND METHODS

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

Mouse and Human Fibroblasts as Feeders

3T3-J2 mouse fibroblasts were kindly provided by Prof. Howard Green (Harvard University, Boston, MA) and maintained in DMEM with 10% adult bovine serum and 1% penicillin/streptomycin (all from Invitrogen). MRC-5 fibroblasts (CCL-171) were purchased from ATCC, 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, CA). Limbal stromal fibroblasts were isolated from a total of 6 donors (age range 26 - 67 year old, 2 male and 4 female, preserved from 7-14 days in Optisol) and grown as previously described. 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 6-9, culture medium was changed three times a week, cultures were passaged upon reaching 70-80% confluence and maintained at 37°C and 20% O_2. All feeders were plated at a density of 2.4×10^4 cells/cm^2 and mitotically inactivated with 4 μg/mL of mitomycin C (MMC, Sigma) for 2 hours at 37°C before seeding limbal epithelial cells (LECs). This concentration of MMC was selected after trial of 1, 4 and 8 μg/mL as the minimum amount needed to arrest cell growth of each type of feeder cells used in these experiments by cell counts 3 days later, similar to prior reports.

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 or the Scheie eye institute after cornea transplantation. Cadaveric tissue was used only for the experiments comparing different isolation methods. For the rest of the experiments, limbal epithelial specimens 1x2mm 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 (Table 1 and Supplemental Table S1). The whole fresh limbal specimen or the limbal rim from cadaveric donors after 8mm 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 (Roche) 1.2 or 2.4 IU at 37°C agitated with an orbital shaker at 90 rpm 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 70μm cell strainer (BD Falcon, USA) in order 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 Y-27632 (Calbiochem), after our preliminary trials of 0, 1 and 10 μl/ml showed improved CFE by about 35% and 15% with addition of 10 and 1 μl/ml Rho inhibitor,

respectively, which were confirmed with recent reports from another group.[43] 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 x 10^4 cells/cm^2 on feeder layer-containing wells. Cell cultures were incubated at 37°C and 95% humidity under 20%, 14% or 5% O_2 with different culture media (Supplemental Table 2 and 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 plate and passaged weekly before confluent at density 1:3 after dissociation with Trypsin 0.25% containing 10µl/ml Y-27632 (Calbiochem) which was also added in the initial culture medium. Cell counts and cell diameter analysis were obtained during passages with Scepter 2.0 Automated Cell Counter with 40µm sensor (Millipore, USA). Cell size was analyzed as cells with diameter up to 10µm (≤10µm) or larger than 10µm, based on prior reports of higher expression of p63α in cells smaller than 10µm. For the colony-forming efficiency (CFE) assay, 500-2,000 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% PFA for 5 min at room temperature, washed with Phosphate Buffered Saline (PBS), stained with 2% rhodamine B (Sigma), photographed with a light box and analyzed with Image J software. Colonies with appearance of holoclones, meroclones and aborted colonies were identified as defined by Green et al. Holoclones were large with area of at least 10mm^2 and smooth perimeter, aborted colonies were smaller than 5 mm^2 containing mostly large and flattened epithelial cells and irregular perimeter, while meroclones were the rest of the colonies typically with wrinkled perimeter. 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% paraformaldehyde for 5 minutes, permealization with 0.3% Triton-X (Sigma) in PBS 3x5 minutes followed by PBS wash and blocking with 1% Bovine Serum Albumin (Sigma) in PBS for 1 hour. Primary antibodies (Supplemental Table S4) for p63α (Cell Signaling), p63 (4A4 Abcam), ABCG2 (Millipore), and C/EBPδ (Santa Cruz), cytokeratin 15 (Covance), cytokeratin 12 (Santa Cruz) and mucin 1 (Santa Cruz) 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) at 1:1,000 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 ether omitted primary or isotype control antibody.

Fluorescence images were acquired with an Olympus IX81 microscope running Metamorph 7 software. Images for quantification were from cytospins and had standardized exposure times for both DAPI and p63α signals. Image analysis was performed with Image J software, by selecting nucleus counter on the DAPI image and Region of Interest (ROI) analysis for acquiring fluorescence intensity on both DAPI and p63α images. A minimum of

500 cells was counted for each specimen. Data were exported to Excel where the ratio of p63α/DAPI intensity was calculated and the distribution of intensity was graphed. In initial experiments, the cell size from bright field images was correlated with p63α, as described by Di Iorio et al, with a yield of high intensity p63α cells between 4 and 9% (5.8 ± 2.5, n=6) for the baseline culture condition, which correlated with intensity ratio of p63α/DAPI of 1.5 or higher, and that ratio was selected as threshold. The percentage of $p63\alpha^{bright}$ cells was multiplied with the total number of cells in each plate to yield absolute number of $p63\alpha^{bright}$ cells.

RNA Extraction, Reverse Transcription, Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and RNeasy Mini and QIA Shredder (Qiagen) according to manufacturer's protocol. RNA quality and quantity were measured using Nanodrop 100 (Thermo Scientific) and Bioanalyser 2100 (Agilent). The High Capacity RNA to cDNA kit (Applied Biosystems) 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 p63α (Hs00978338_m1), ABCG2 (Hs01053796_m1), C/EBPδ (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 was carried out using SAS 9.2 software (GraphPad, USA). One-way analysis of variance (ANOVA) with Bonferroni *post hoc* tests was used, as well as Spearman correlations. All error bars represent standard error of the mean 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 old (range 18 – 75), were stored in Optisol for an average of 13 ± 6 days (range 3 – 35) prior to isolation and were used for evaluation of a total of 15 variations of dissociation methods (Supplemental Table S1) with 6 representative variations (3 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 Colony Forming Efficiency (CFE, Supplemental Figure S1). Methods that had been previously reported, namely trypsin 0.05% for 80 minutes, dispase 1.2 IU/ml for 2 hours, and dispase 2.4 IU/ml for 1.5 hour followed by scraping and trypsin 0.25% for 10 minutes, 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 number 3 being the best among the three. Twelve variations of method 3 were then tried, adjusting the concentration and exposure time of dispase and trypsin/TrypLE (Table S1 and Fig. S1) and the three best performing methods are shown in Fig 2 as methods number 4, 5, and 6..

Statistical analysis of all isolation methods showed that yield with isolation methods 1, 2 and 3 was significantly lower than several other methods, including methods 5 and 6 (p=0.0001, Fig 2 and S1). No significant difference was seen for viability among the different methods varying between 72% and 93% (p=0.1635, Fig. S1B). CFE with gentle trypsinization methods 5 and 6 was significantly higher than methods 1-3 and several variations of method 3 (p=0.00001, Fig 2 and 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, scrapping 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 yield of 1,352,687 ± 47,814 cells/whole sclerocorneal donor rim, viability of 90 ± 2 % and CFE of 0.077 ± 0.02 % (mean ± SEM, Fig. 2 and 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 old (range 25-90) with this isolation method, showed yield of 305,857 ± 59,657 cells/mm^2, viability of 92 ± 2 %, CFE of 2 ± 0.5 % and percentage of aborted colonies 8 ± 3 % (mean ± SEM).

Xeno-free media and oxygen tension with murine feeders

Limbal epithelium specimens from 23 patients of mean age 70 ± 11 years old (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 were then seeded in parallel on 3T3-J2 feeders and cultured with different media (see Supplemental Table S2 for complete list of media) under 20%, 14% or 5% O_2 (Supplemental Fig. S2). X-KGM medium at 20% O_2 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 Holoclone Forming Efficiency (HFE) at initial seeding. A maximum of 10 weekly serial passages was noted under certain conditions. 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. S2A-B). Multivariate analysis of effect of donor age, gender, 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 gender (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. S2G-H) while the rest of the media were not significantly different from the baseline value of 8%. CFE was higher for X-SHEM and XF-SHEM media under 14% O_2 only (p=0.003, Fig. S2C-D) while HFE was higher for X-SHEM at 14% O_2 and XF-SHEM at 20% and 14% O_2 (p=0.009 Fig. S2E-F). Low (0.1mM) calcium Xeno-free medium at 20% O_2 supported more passages than the higher concentrations of 0.4, 1.05 and 1.3 mM when EGF was 10 ng/ml (p=0.0001, Fig. S2A) and had low percentage of aborted colonies (S2G). 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 S2B). The number of small cells (≤10μm) during passages showed no statistically significant difference among different conditions, but conditions with less passages tended to show lower number of small cells (Supplemental Figure 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 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 p63α^{bright} cells quantified at the first passage showed more p63α^{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. S3A) but no significant difference among the rest of the media and the baseline condition which had an average number of 8 ± 2.3 % of p63α^{bright} cells cultured with X-KGM.

Immunocytochemistry expression for limbal stem cell markers was high for p63α, ABCG2, C/EBPδ and K15 and low or absent for K12 and MUC1 at the first passage for all conditions evaluated and we found it difficult to predict the amount of passages from evaluating LSC marker expression under the microscope, except in extreme cases of only 1-2 passages were we could see more K12 expression and less p63α^{bright} cells at P1. The quantified p63α^{bright} expression in cytospins showed a positive correlation with the number of passages that was close but not statistically significant (rho 0.34, p=0.063). The RT-PCR ratio of p63α/K12 differentiation marker expression showed no significant difference among all the media used (p=0.79, Fig. S3B).

Oxygen tension of 20% supported more passages with low calcium xeno-free medium, while 5% O_2 supported more passages with baseline X-KGM media (p=0.0001). Xeno-free medium under 20% O_2 also showed more p63α^{bright} cells with immunocytochemistry (p=0.01) and no significant difference in RT-PCR ratio p63α/K12 or aborted colonies (Fig. S2G). Univariate analysis on p63α^{bright} cells showed higher expression in 20% than 14% or 5% O_2 (p=0.031), while there was no significant effect of oxygen on RT-PCR ratio of p63α/K12 (p=0.12).

Xeno-Free medium with low calcium 0.1mM and EGF 10ng/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) or neonatal (N-HDF) or 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 ± 4 (range 25-87) years old, 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 0.1mM calcium and 10ng/ml EGF and grown under 20% or 5% O_2. The condition of 3T3-J2 feeders with X-KGM at 20% O_2 was the baseline condition included in every experiment.

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). MRC-5, F-HDF and A-HDF feeders with Xeno and Xeno-free media and N-HDF with Xeno media showed more p63α^{bright} cells (p=0.022) and higher RT-PCR ratio p63α/K12 (p=0.03). A-HDF could not support more than 3 passages of LECs in culture, while MRC-5, F-HDF and 3T3-J2 supported at least 8 passages and N-HDF supported about 5 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% O_2 (p=0.35). MRC-5 and F-HDF feeders at 20% O_2 were selected for further analysis.

Colonies grown on MRC-5 feeders with Xeno-free medium at 20% O_2 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. 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 (Supplemental Fig. S4, results from MRC-5 and baseline condition shown). CFE showed a gradual decrease during multiple passages that was not statistically different between MRC-5, F-HDF feeders and baseline condition (Supplemental Fig. S5, results from MRC-5 and baseline condition shown). Colonies on all feeders showed good expression of p63α at P1 that lasted throughout multiple passages. RT-PCR ratio of p63α/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. Colonies grown on MRC-5, F-HDF and 3T3-J2 feeders with Xeno-free medium demonstrated high expression of p63α, ABCG2, C/EBPδ and K15 with minimal or no expression of cornea (K12) and conjunctiva (MUC1) differentiation markers from the first passage (Supplemental 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. RT-PCR for p63α, ABCG2, C/EBPδ, Bmi1 and K12 during multiple passages showed robust upregulation of mostly p63α 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. A summary of all the results obtained is presented in Supplemental Table S5.

Overall, MRC-5 feeders with Xeno-free low calcium medium at 20% O_2 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.

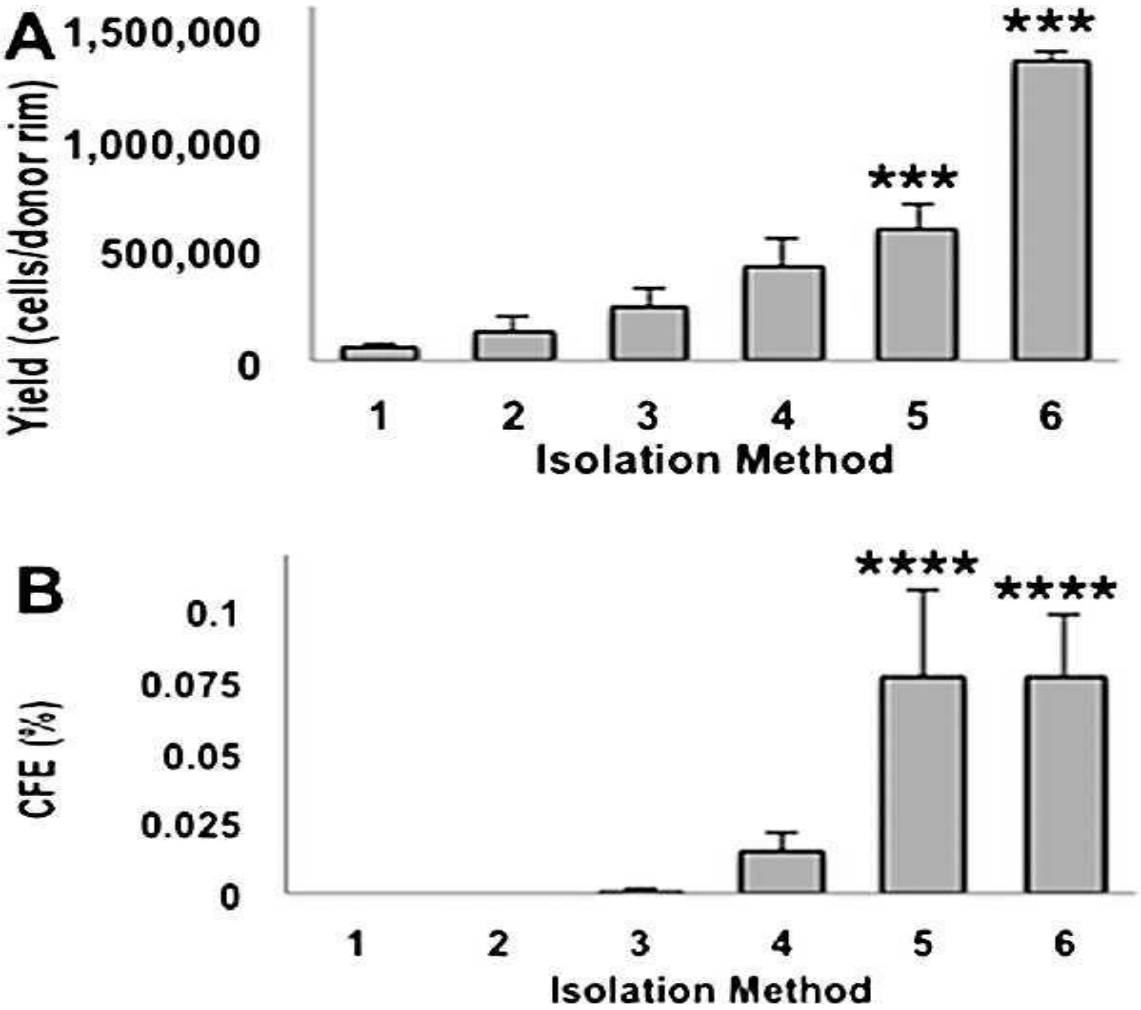
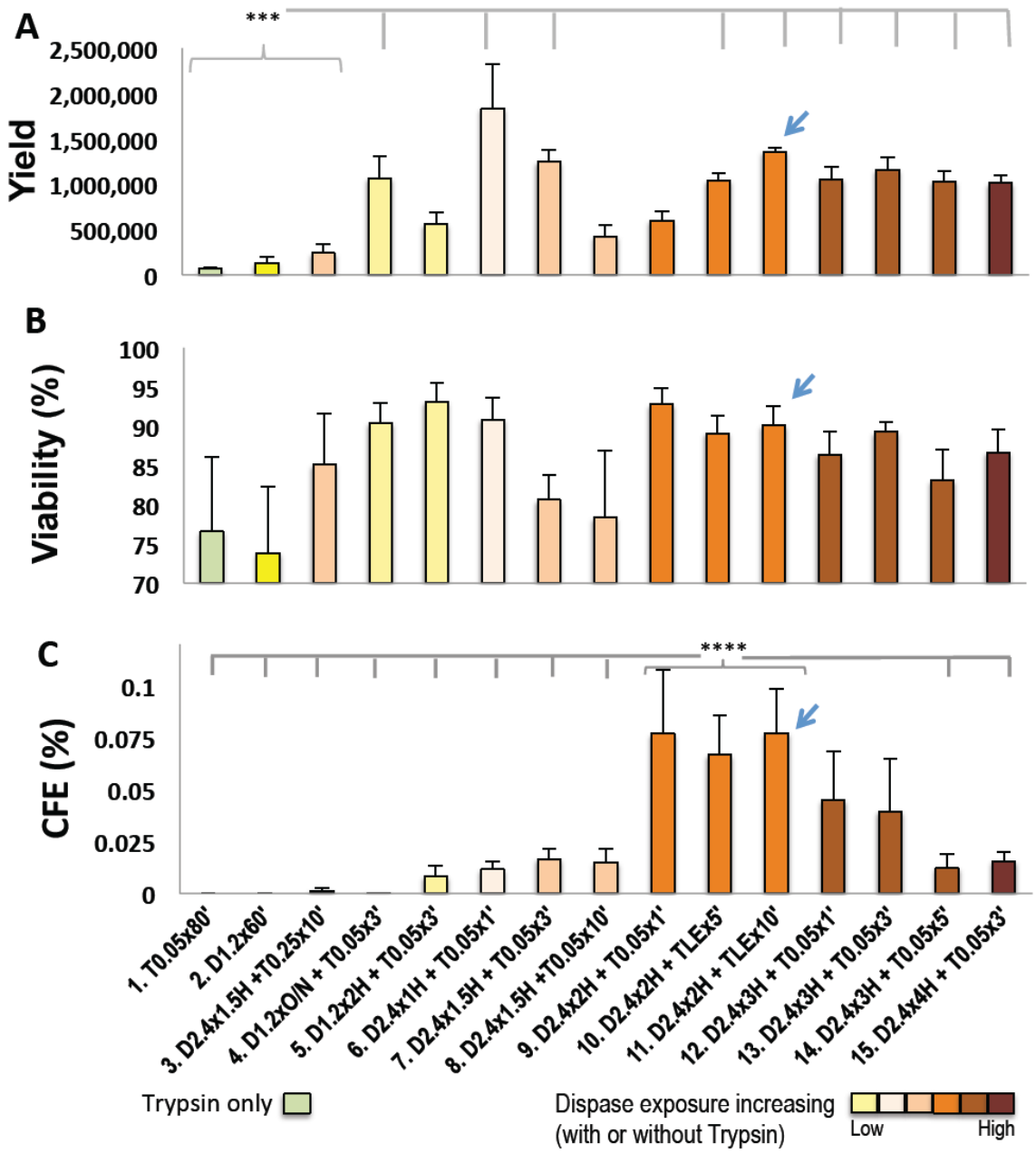


Figure 2. Selection of Isolation method based on Yield and Colony Forming Efficiency (CFE). Results of 6 selected isolation methods (Table 1) out of the total of 15 variations are shown, three of them previously published (number 1-3) and 3 variations of method number 3 (number 4-6). They were evaluated 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 6-15 different limbal specimens derived from a total of 139 donors. Asterisks note statistically significant difference of p=0.0001 or p=0.00001(****) with ANOVA/Bonferoni tests between methods 5, 6 and methods 1, 2, 3 in (A) and methods 1, 2, 3, 4 in (B). Method 6 (Dispase 2.4 IU/ml for 2 hours and TLE for 10 minutes) consistently showed the best results**.**

Supplemental Table S1. Complete list of isolation methods.

Number	Isolation method	Abbreviation	Donors (N)
1	Trypsin 0.05% x 80 min (4 cycles of 20 minutes)	T0.05x80'	15
2	Dispase 1.2 IU/ml x 2hrs	D1.2x60'	12
3	Dispase 2.4 IU/ml x 1.5hr and Trypsin 0.25% x 10min	D2.4x1.5H +T0.25x10'	7
4	Dispase 1.2 IU/ml overnight and Trypsin 0.05% x 3min	D1.2xO/N + T0.05x3'	9
5	Dispase 1.2 IU/ml x 2hrs and Trypsin 0.05% x 3min	D1.2x2H + T0.05x3'	9
6	Dispase 2.4 IU/ml x 1hr and Trypsin 0.05% x 1min	D2.4x1H + T0.05x1'	7
7	Dispase 2.4 IU/ml x 1.5hr and Trypsin 0.05% x 3min	D2.4x1.5H + T0.05x3'	6
8	Dispase 2.4 IU/ml x 1.5hr and Trypsin 0.05% x 10min	D2.4x1.5H +T0.05x10'	7
9	Dispase 2.4 IU/ml x 2hrs and Trypsin 0.05% x 1min	D2.4x2H + T0.05x1'	10
10	Dispase 2.4 IU/ml x 2hrs and TLE x 5min	D2.4x2H + TLEx5'	7
11	Dispase 2.4 IU/ml x 2hrs and TLE x 10min	D2.4x2H + TLEx10'	7
12	Dispase 2.4 IU/ml x 3hrs and Trypsin 0.05% x 1min	D2.4x3H + T0.05x1'	10
13	Dispase 2.4 IU/ml x 3hrs and Trypsin 0.05% x 3min	D2.4x3H + T0.05x3'	15
14	Dispase 2.4 IU/ml x 3hrs and Trypsin 0.05% x 5min	D2.4x3H + T0.05x5'	9
15	Dispase 2.4 IU/ml x 4hrs and Trypsin 0.05% x 3min	D2.4x4H + T0.05x3'	9

Notes: Methods 8, 9 and 11 were renamed 4, 5 and 6, respectively, in the main text.

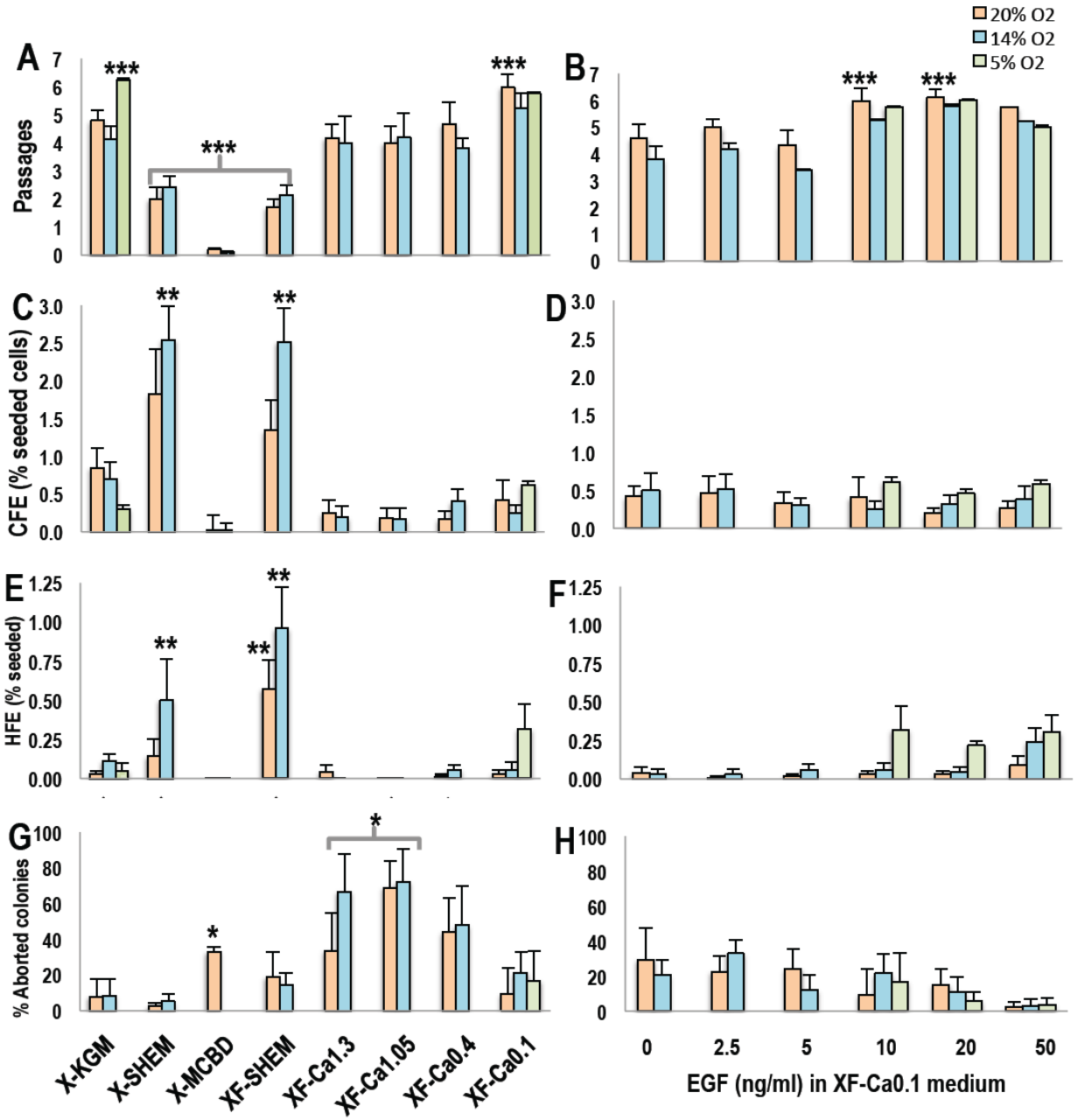


Supplemental Figure S1. Effects of different isolation methods on Yield, Viability and Colony Forming Efficiency (CFE). Fifteen isolation methods (Table S1), three of them previously published (number 1-3) and 12 variations of method number 3 (in different shades of yellow-brown based on increased exposure to dispase) were evaluated for yield as total number of cells isolated from a cadaveric donor corneoscleral rim (**A**), viability with trypan blue stain (**B**) and CFE (**C**). Each bar represents mean and SEM from 6-15 different limbal specimens derived from a total of 139 cadaveral donors. D: Dispase of 1.2 or 2.4 IU/ml, T: Trypsin, TLE: TrypLE. Asterisks note significant difference of p=0.0001(***) or p=0.00001(****) with ANOVA. Arrow notes the method that was selected for further experiments.

Supplemental Table S2. List of culture media and abbreviations used

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
X-SHEM	No	1.05	10	DMEM-F12 1:1	Yes
X-MCBD	No	0.03	10	MCDB151	Yes
XF-SHEM	Yes	1.05	10	DMEM-F12 1:1	No
XF-Ca1.3	Yes	1.3	10	DMEM-F12 2:1	No
XF-Ca1.05	Yes	1.05	10	DMEM-F12 1:1	No
XF-Ca0.4	Yes	0.4	10	DMEMnoCa-F12-DMEM 3:2:1	No
XF-Ca0.1-EGF10	Yes	0.1	10	DMEMnoCa-F12 2:1	No
XF-Ca0.1-EGF0	Yes	0.1	0	DMEMnoCa-F12 2:1	No
XF-Ca0.1-EGF2.5	Yes	0.1	2.5	DMEMnoCa-F12 2:1	No
XF-Ca0.1-EGF5	Yes	0.1	5	DMEMnoCa-F12 2:1	No
XF-Ca0.1-EGF20	Yes	0.1	20	DMEMnoCa-F12 2:1	No
XF-Ca0.1-EGF50	Yes	0.1	50	DMEMnoCa-F12 2:1	No

Abbreviations: X and XF for Xeno and Xeno-Free media, respectively; KGM: Keratinocyte Growth Medium; SHEM: Supplemented Hormonal Epithelial Medium; EGF: Epidermal Growth Factor. Detailed formulations in Supplemental Table S3.

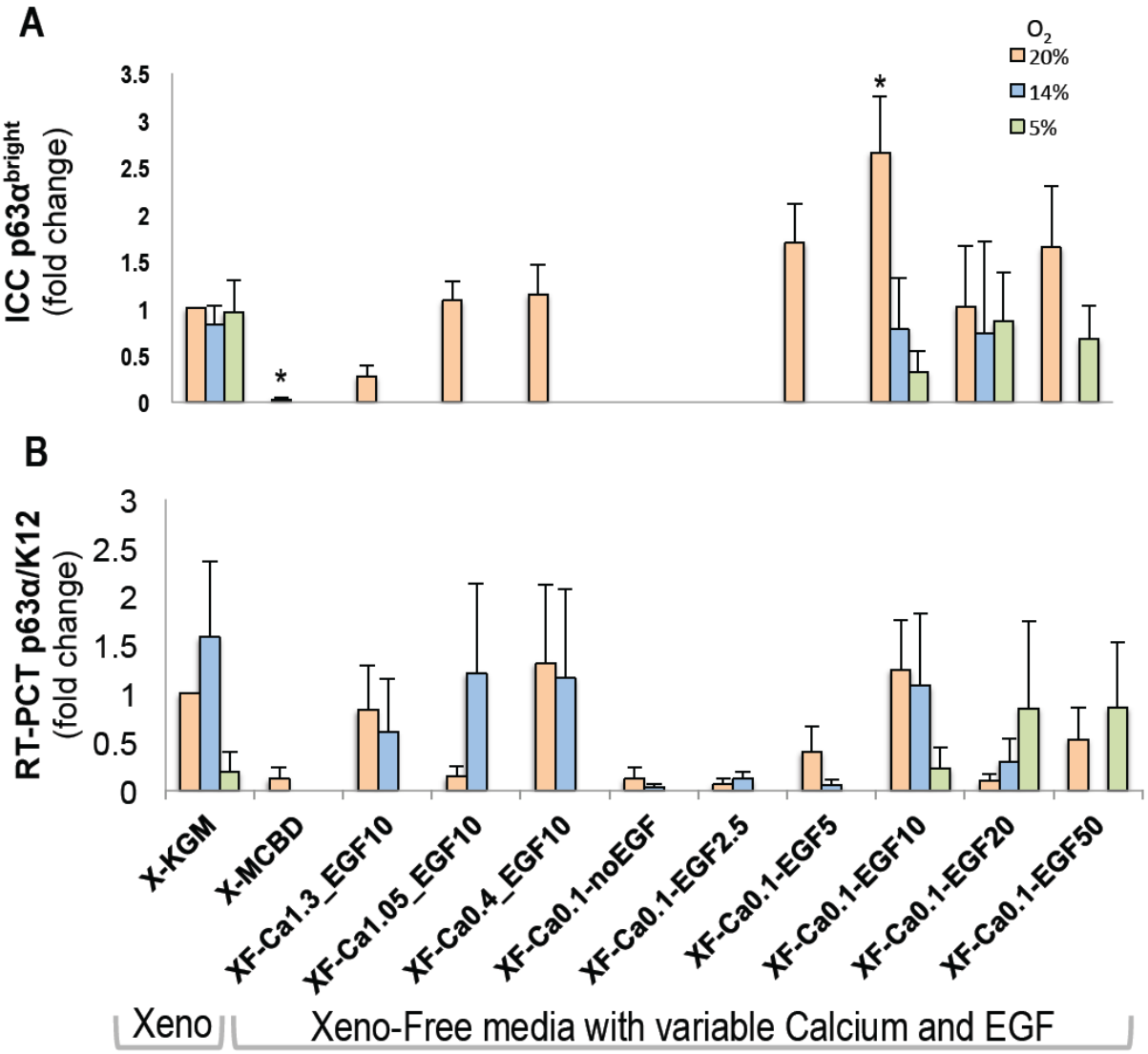


Supplemental Figure S2. Detailed effect of media and oxygen tension on LEC passages and clonogenicity. Xeno (X-KGM, X-SHEM and X-MCBD) and Xeno-Free media (XF-SHEM and XF-Ca1.3, XF-Ca1.05, XF-Ca0.4, XFCa0.1 with decreasing concentrations of calcium) evaluation with outcomes: number of weekly serial passages till senescence (**A-B**), CFE (**C-D**), Holoclone Forming Efficiency (HFE, **E-F**), and percentage of aborted colonies (**G-H**), under 20% O_2 (orange bars), 14% (blue bars) or 5% (green bars). Xeno-free medium with Calcium 0.1mM (XF-Ca0.1) showed consistent support of multiple passages (**A**) and was further evaluated using different concentrations of EGF 0-50 ng/ml (**B**). X-KGM medium at 20% oxygen was included in every experiment as baseline condition and LECs were seeded at the same density in all conditions and grew in parallel. Bars represent means and SEM from 6-23 fresh limbal specimens. X-KGM at 20% O_2 was included in every experiment as baseline. Asterisks note significant difference of p=0.0001 (***) for passages, p=0.003 (**) for CFE, p=0.009 for HFE (**), and p=0.019 (*) for percentage of aborted colonies with ANOVA among all media variations under 20%, 14% and 5% O_2.

Supplemental Table S3. Composition of different culture media of LECs.

Culture Medium Abbreviation	X-KGM	X-SHEM	X-MCBD	XF-SHEM	XF-Ca1.3	XF-Ca1.05	XF-Ca0.4	XF-0.1
Calcium concentration	1.3 mM	1.05 mM	0.03 mM	1.05 mM	1.3 mM	1.05 mM	0.4 mM	0.1 mM
Base medium	DMEM-F12 2:1	DMEM-F12 1:1	MCDB151	DMEM-F12 1:1	DMEM-F12 2:1	DMEM-F12 1:1	DMEMnoCa-F12-DMEM 3:2:1	DMEMnoCa-F12 2:1
Fetal Calf Serum (10437, Gibco)	10%	5%						
Human AB serum (100-512, Gemcell)				5%	5%	5%	5%	5%
Cholera Toxin (C8052, Sigma)	0.1nM	1 nM						
Isoproterenol (I5627, Sigma or ITS)				0.5 µg/ml	0.5 µg/ml	0.5 µg/ml	0.5 µg/ml	0.5 µg/ml
Insulin (HR, 91077C, Sigma or ITS 354351 BD)	5 µg/ml	5 µg/ml	5 µg/ml*	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml
Transferrin (ITS, 354351 BD)		5 µg/ml	5 µg/ml*	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml
Selenous acid (ITS, 354351 BD)		5 ng/ml		5 ng/ml	5 ng/ml	5 ng/ml	5 ng/ml	5 ng/ml
Hydrocortisone (H0888, Sigma)	0.4 µg/ml	5 µg/ml	0.18 µg/ml*	5 µg/ml	0.4 µg/ml	0.4 µg/ml	0.4 µg/ml	0.4 µg/ml
Adenine (A2786, Sigma)	0.18 mM				0.18 mM	0.18 mM	0.18 mM	0.18 mM
Triodothyronine (IRMM469, Sigma)	2 nM				2 nM	2 nM	2 nM	2 nM
Glutamine (GlutaMAX Invitrogen)	4 mM				4 mM	4 mM	4 mM	4 mM
Pyruvate sodium (11360, Invitrogen)					1mM	1mM	1mM	1mM
MEM NEAA (11140, Invitrogen)					1mM	1mM	1mM	1mM
Albumin (human A6784, Sigma)					500 mg/L	500 mg/L	500 mg/L	500 mg/L
HCGS (S-009-05, Gibco), BPE			0.2%*					
Pen Strep (15140, Invitrogen)	50 IU/ml	50 IU/ml	50 IU/ml	50 IU/ml	50 IU/ml	50 IU/ml	50 IU/ml	50 IU/ml
Amphotericin B (15290-018, Invitrogen)	0.25 µg/ml	0.25 µg/ml	0.25 µg/ml	0.25 µg/ml	0.25 µg/ml	0.25 µg/ml	0.25 µg/ml	0.25 µg/ml
EGF (HR, 324831, Calbiochem)	10 ng/ml	10 ng/ml	10 ng/ml	10 ng/ml	10 ng/ml	10 ng/ml	10 ng/ml	0 to 50 ng/ml

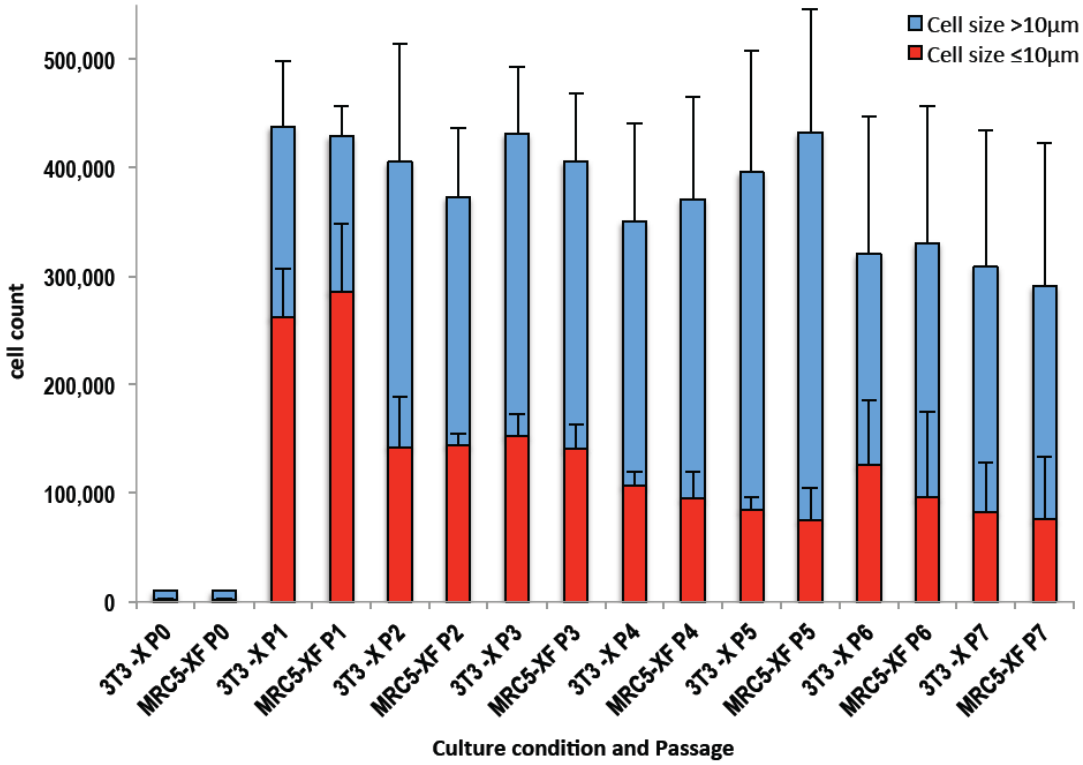
Note: Basal media were composed from DMEM 11965, DMEM without Calcium 21068, F12 11765 (all three from Invitrogen) and MCBD151 from Sigma. HR: Human recombinant, EGF: Epidermal Growth factor. Asterisk (*) notes components of HCGS supplement which contains Bovine Pituitary Extract (BPE), bovine insulin, hydrocortisone, bovine transferrin and mouse EGF.



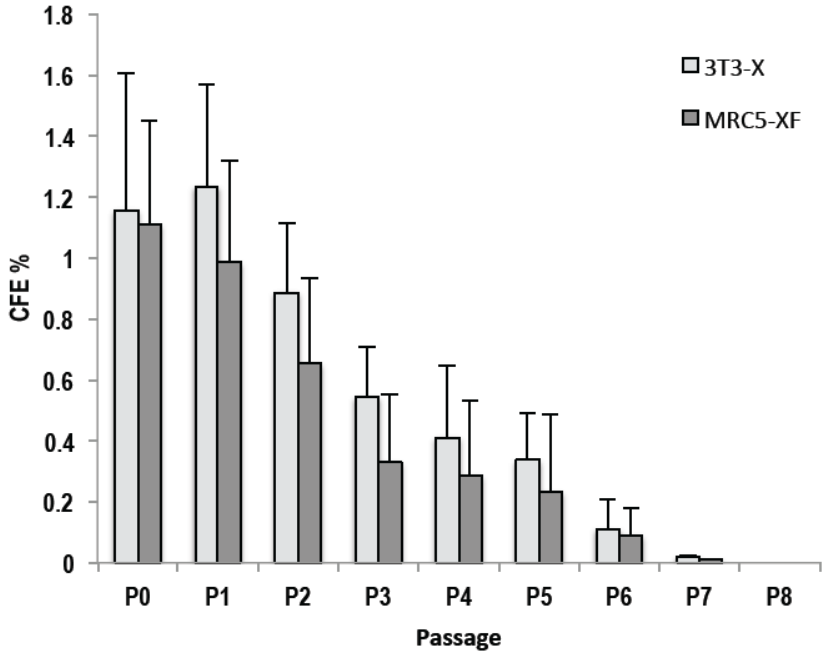
Supplemental Figure S3. Effect of media and oxygen tension on expression of LSC markers. Two Xeno (X-KGM and X-MCBD) media and 9 variations of Xeno-Free media with increasing concentrations of Calcium and EGF: were evaluated under different Oxygen tensions: 20% (orange bars), 14% (blue bars) or 5% (green bars). All data were normalized to the medium X-KGM at 20% oxygen that was included in every experiment as baseline and LECs were seeded at the same density in all conditions and grew in parallel. Immunocytochemistry for p63α^{bright} cells showed higher expression in low calcium medium with 10ng/ml EGF medium and lower expression with X-MCBD medium at 20% O_2 (**A**). RT-PCR ratio for p63α/K12 did not show statistically significant differences in the different media (**B**). Each bar represents mean and SEM from 6-14 fresh limbal specimens. Asterisk notes significant difference of p=0.01 with ANOVA between all media variations under all oxygen tensions (20%, 14% and 5%). EGF: Epidermal Growth Factor.

Supplemental Table S4. List of primary antibodies used for immunocytochemistry.

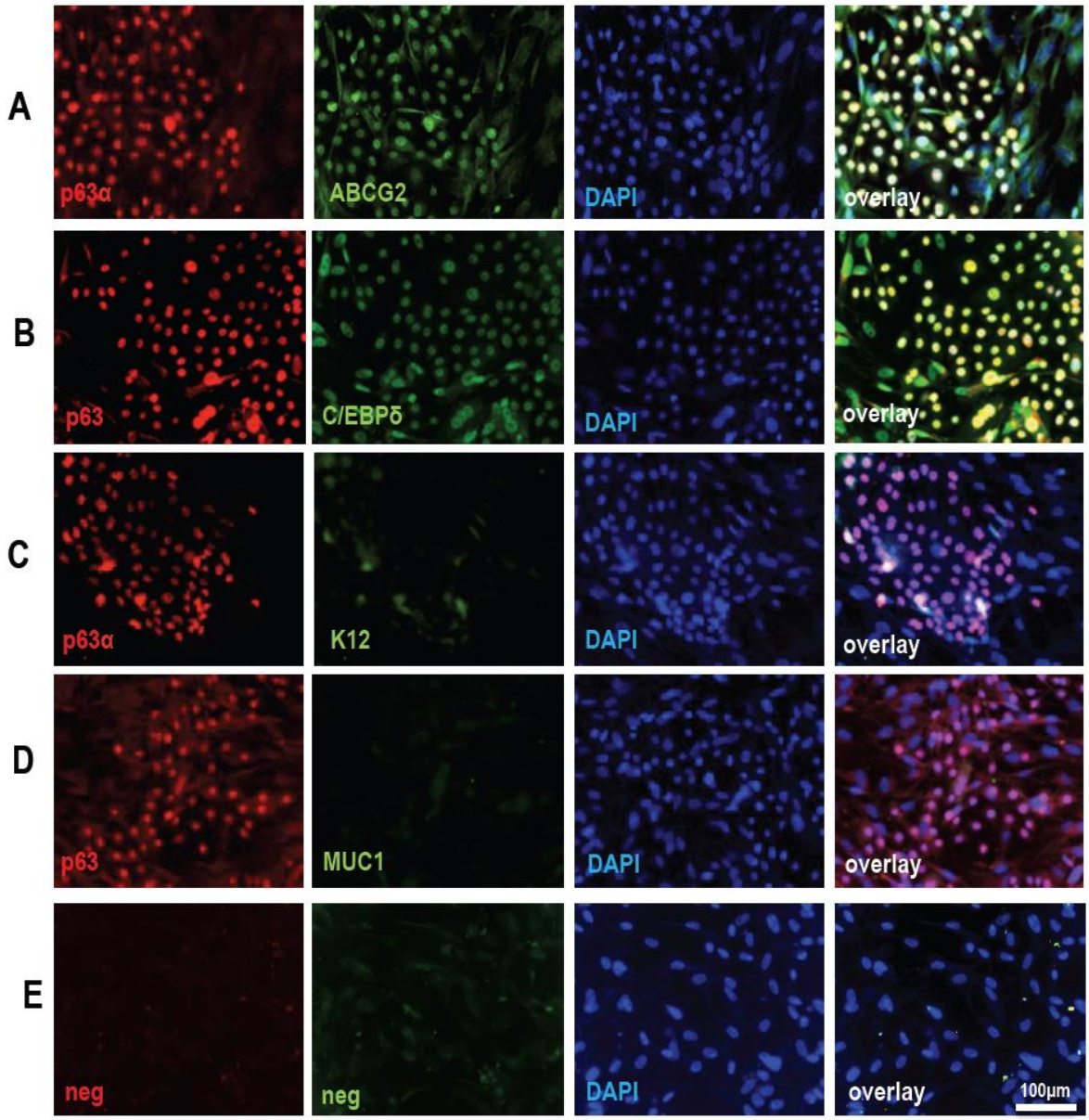
Primary antibodies	Clone	Host	maker	catalog #	Dilution
p63α		rabbit	Cell Signaling	4892	1:100
p63	4A4	mouse	Abcam	3239	1:100
ABCG2	BXP-21	mouse	Millipore/Chemicon	MAB4146	1:100
C/EBPδ	C-22	rabbit	Santa Cruz	sc-151	1:100
CK-15		chicken	Covance	PCK-153P	1:100
CK-12	L-20	goat	Santa Cruz	17099	1:100
MUC1	H-265	rabbit	Santa Cruz	15333	1:100



Supplemental Figure S4. Cell size analysis during multiple passages in baseline condition of 3T3-J2 feeders with Xeno (X) medium and MRC-5 feeders with Xeno-Free (XF) medium. Cell size was analyzed as cells with diameter up to 10µm (≤10µm, **red**) or larger than 10µm (**blue**). Limbal epithelial cells from fresh specimens were seeded in parallel as 10,000 cells per well (with an average of 2,000 cells of ≤10µm diameter) in different culture conditions and passaged at 1:3 weekly. Bars represent mean and SEM of four replicates.



Supplemental Figure S5. Colony Forming Efficiency (CFE) with serial passages. CFE gradually decreased with serial passages of fresh limbal epithelial cells cultivated under baseline condition of 3T3-J2 feeders with Xeno-KGM (X) medium and with MRC-5 feeders and Xeno-Free (XF) medium. Bars represent mean and SEM of four replicates.



Supplemental Figure S6. LECs express appropriate markers of LSCs in first passage under Xeno-free culture conditions. Colonies expressed limbal progenitor cell marker p63α or p63 (red, **A-D**) as well as ABCG2 (green, **A**) and C/EBPδ (green, **B**) but not differentiation marker cytokeratin 12 (K12) for cornea (green, **C**) or mucin1 (MUC1) for conjunctiva (green, **D**) differentiated epithelial cells, and were counterstained with DAPI (blue, **A-D**) for overlays (**A-D**). Representative negative control for each channel (**E**).

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one

publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Optimal isolation and xeno-free culture conditions for limbal stem cell function.	Stasi K, Goings D, Huang J, Herman L, Pinto F, Addis RC, Klein D, Massaro-Giordano G, Gearhart J	Investigative Ophthalmology and Visual Science (IOVS)	May 2013	☐Submitted ☐Accepted ☒Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____ No____X____

If yes, please describe your plans:

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

The research had an impact on selecting the best culture method for future extension to a clinical trial for treatment of limbal stem cell deficiency.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were

no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

This was 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 p63αbright cells and percentage of aborted colonies. Our results show that certain Xeno-free conditions can support limbal epithelial stem cell culture under Good Manufacturing Practices facilitating translation into clinical applications.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No____X____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____X____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

John D. Gearhart, Ph.D., is the James W. Effron University Professor and is the Director of the Institute for Regenerative Medicine at the University of Pennsylvania. He is a developmental geneticist and his research over the past several decades has been directed at an understanding the molecular and cellular basis of human embryonic development. Dr. Gearhart is a leader in the development and use of human reproductive technologies, embryo and germ cell manipulations and in the genetic engineering of cells. In 1998, Dr. Gearhart and his research team at Johns Hopkins published the first report on the derivation of pluripotent stem cells from germ cells of the human embryo. These cells have the capacity to form all cell types and tissues present in the human body and are considered a major starting point for the development of a wide variety of cell-based therapies in the new field of regenerative medicine. His research is focused on the basic science of stem cells, stem cell specialization, and the generation of cell-based therapies for a number of diseases and injuries.

Dr. Gearhart was a founding member of the International Society for Stem Cell Research and serves on a number of advisory boards and committees of foundations, institutes and professional societies involved in stem cell research and policy and science outreach and has served as a consultant or expert witness for many governmental agencies, in states, at the national level and to governments of foreign countries. He currently serves on the FDA Advisory Committee for Cellular, Tissue and Gene Therapies and the World Anti-Doping Authority, Gene Doping Expert Group.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Stasi, Kalliopi	Instructor of Ophthalmology
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING (in chronological order)

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Patras School of Medicine, Patras Greece	M.D.	1985-1991	Medicine
University of Patras School of Medicine, Patras Greece	Ph.D.	1995-2001	Neuroscience

A. Positions and Honors.

Positions and Employment

1992-1992 Internship General Surgery, General Hospital "Pammakaristos", Athens Greece
1992-1994 General Medicine Public Service, Lesvos Island, Greece
1994-1995 Ophthalmology Residency, General Hospital of Florina, Florina Greece
1997-2000 Ophthalmology Residency, General Hospital "Pammakaristos", Athens Greece
2000-2001 Private Practice General Ophthalmology, Athens, Greece
2002-2004 Postdoc Research Fellow, Ophthalmology, Mount Sinai School of Medicine, NewYork, NY
2004-2005 Internship Internal Medicine, Montefiore Hospital, Albert Einstein Sch. of Medicine, New York
2005-2008 Ophthalmology Residency, University of Rochester Eye Institute, Rochester, NY
2008-2009 Cornea and Refractive Surgery Fellow, Wilmer Eye Institute, Johns Hopkins, Baltimore, MD
2009-2013 Instructor of Ophthalmology, Scheie Eye Institute, University of Pennsylvania and K12 Institutional Clinician Scientist Training Award Recipient (75% Research/Training and 25% Clinical)
2013-2013 Assistant Professor of Ophthalmology, Scheie Eye Institute, University of Pennsylvania and K12 Institutional Clinician Scientist Training Award Recipient (75% Research/Training and 25% Clinical)
2013-present Translational Medicine Expert, Novartis Institutes for Biomedical Research
2013-present Adjunct Assistant Professor of Ophthalmology, Scheie Eye Institute, University of Pennsylvania

Honors

2005 Association for Research in Vision and Ophthalmology (ARVO) Foundation/Retina Research Foundation/Joseph M. and Eula C. Lawrence Travel Grant
2006 Heed Ophthalmic Foundation Residency Retreat Award
2007 First Author of paper awarded The New York Academy of Medicine Lewis Rudin Glaucoma Award
2007 Walsh Meeting Invited Talk at NANOS Neuro-ophthalmology meeting
2008 The Ronald G. Mitchels Wilmer Research Grant Award, Johns Hopkins
2008 Aliki Peroti Scholarship Recipient at Cornea Department of Wilmer Eye Institute, Johns Hopkins

B. Selected peer-reviewed publications (in chronological order).

1. Tsourinakis T., Mitsacos A., Voukelatou G., Aletras A., Dermon C., Stasi K., and Kouvelas E.D. Immunohistochemical and autoradiographic studies of the glutamatergic system in chick brain. Abstracts of 12th Annual Meeting of European Neuroscience Association. *European Journal of Neuroscience*, Suppl. 2:18, 1989
2. Mitsacos A., Dermon C.R., Stassi K. and Kouvelas E.D. Localization of L-glutamate binding sites in chick brain by quantitative autoradiography. *Brain Research* 513, 348-352, 1990
3. Stasi K., Mitsacos A.,Triarhou L.C., and Kouvelas E.D. Cerebellar grafts partially reverse amino acid receptor changes observed in the cerebellum of mice with hereditary ataxia: quantitative autoradiographic studies. *Cell Tranplantation* 6, 347-359, 1997
4. Mitsacos A., Stasi K., Tomiyama M., Giompres P., Kouvelas E.D., Cortes R., Palacios J.M., Mengod G. and Triarchou L.C. Nigral transplants in the weaver mouse neostriatum: levels of glutamate and GABA binding sites and AMPA receptor subunit mRNAs. Abstracts of Forum of European Neuroscience. *European Journal of Neuroscience* 10:14310, 1998
5. Stasi K., Tomiyama M., Mitsacos A., Giompres P., Kouvelas E.D., Cortes R., Palacios J.M., Mengod G. and Triarchou L.C. Nigral transplants in the weaver mouse neostriatum: localization of excitatory and inhibitory aminoacid binding sites and AMPA receptor subunits mRNAs. Abstracts of 5th Conference of American Society for Neural Transplantation. *Experimental Neurology* 153(2):380, 1998
6. Triarchou L.C., Lee W.H., Zhang W., Stasi K., Mitsacos A., and Kouvelas E.D. Functional aspects of cerebellar transplantation in mice with hereditary degenerative ataxia. Abstracts of Experimental Biology Meeting. *The Federation of American Societies for Experimental Biology (FASEB) Journal* 13(5):A710, 1999
7. Triarchou L.C., Stasi K., Mitsacos A., Giompres P. and Kouvelas E.D. Functional nigral cell grafting and striatal amino acid receptors in a mouse model of Parkinson's disease. Abstracts of Experimental Biology Meeting. *The Federation of American Societies for Experimental Biology (FASEB) Journal* 13(5):A710, 1999
8. Mitsacos A., Tomiyama M., Stasi K., Giompres P., Kouvelas E.D., Cortes R., Palacios J.M., Mengod G., and Triarhou L.C. [^{3}H]CNQX and NMDA-sensitive [^{3}H]glutamate binding sites and AMPA receptor subunit RNA transcripts in the striatium of normal -and weaver mutant mice and effects of ventral mesencephalic grafts. *Cell Tranplantation* 8, 11-23, 1999
9. Stasi K., Mitsacos A., Giompres P., Kouvelas E.D., and Triarhou L.C. Partial restoration of striatal GABA-A receptor balance by functional mesencephalic dopaminergic grafts in mice with hereditary Parkinsonism. *Experimental Neurology* 157, 259-267, 1999
10. Stasi K., Naskar R., Thanos S., Kouvelas E.D. and Mitsacos A. Glutamate and GABA receptor subtypes in the RCS rat retina. *Graefes Archives of Clinical and Experimental Ophthalmology*, 241(2):154-60, 2003
11. Armata I., Giompres P., Smith A., Stasi K., Kouvelas E.D. and Mitsacos A. Genetically induced retinal degeneration leads to changes in metabotropic glutamate receptor expression. Neurosci Lett. 393(1):12-7, 2006. Epub 2005 Oct 6
12. Stasi K., Paccione J., Bianchi G., Friedman A., Podos S.M. and Danias J. Photodynamic treatment in a rabbit model of glaucoma surgery. *Acta Ophthalmologica Scandinavica* 84(5): 661-6, 2006
13. Stasi K., Nagel D., Yang X., Wang RF., Ren L., Podos SM., Mittag T. and Danias J. Complement component 1Q (C1Q) upregulation in retina of murine, primate and human glaucomatous eyes. *IOVS* 47(3): 1024-9, 2006

14. Stasi K., Nagel D., Yang X., Ren L., Mittag T. and Danias J. Ceruloplasmin upregulation in retina of murine and human glaucomatous eyes. *IOVS* 48(2): 727-32, 2007
15. Stasi K., Rao NA, Feldon SE and DiLoreto DA. Episodes of Amaurosis Fugax Secondary to Eosinophilic Vasculitis in an AIDS Patient. J. Neuroophthalmology 29(1):58-61, 2009.
16. Stasi K. Chuck RS. Update on Phototherapeutic Keratectomy. Current Opinion Ophthalmology. Current Opinion in Ophthalmology, 20(4):272-5, 2009.
17. Stasi K, Goings D, Huang J, Herman L, Pinto F, Addis RC, Klein D, Massaro-Giordano G, Gearhart JD. Optimal Isolation and Xeno-free culture conditions for limbal stem cell function. IOVS 55(1):375-86, 2014

C. Research Support

Aliki Peroti Scholarship and The Ronald G Mitchels 2008 Wilmer Research Grant Award
Stasi, Kalliopi 7/1/08 - 7/1/2009
Identification and characterization of cultured human limbal stem cells with live non-invasive autofluorescence evaluation through Redox Fluorometry.
Faculty Mentor: Roy S. Chuck M.D., Ph.D., Wilmer Eye Institute
This study evaluated a non-invasive way to identify live cultured limbal stem cells that can finally be used for transplantation in a variety of patients with stem cell deficiency of the cornea.
Role: Principal Investigator

K12 Institutional Mentored Clinician Scientist Development Program Award
Stasi, Kalliopi 10/2009 - 9/2013
Evaluation of stem and progenitor cells for the treatment of limbal stem cell deficiency.
This study evaluates the use of cultivated limbal epithelial stem cells and progenitor cells, as well as similar cells derived for induced pluripotent stem cells, for the treatment of limbal stem cell deficiency on an organ culture model of the disease.
Role : Principal Investigator

Pennsylvania Commonwealth Universal Research Enhancement (CURE) Program
SAP#4100050912 4/2012 - 9/2013
The objective of this project was to develop and directly compare culture conditions of LSCs for use in future clinical transplantation in patients with LSCD.
Role: Co-Investigator