

Wnt Signaling Is Required for the Maintenance of Human Limbal Stem/Progenitor Cells In Vitro

Sheyla González, Denise Oh, Elfren R. Baclagon, Jie J. Zheng, and Sophie X. Deng

Stein Eye Institute, University of California, Los Angeles, California, United States

Correspondence: Sophie X. Deng, Stein Eye Institute, University of California, 100 Stein Plaza, Los Angeles, CA, USA; deng@jsei.ucla.edu.

Jie J. Zheng, Stein Eye Institute, University of California, 100 Stein Plaza, Los Angeles, CA, USA; jzheng@jsei.ucla.edu.

Submitted: September 24, 2018

Accepted: November 30, 2018

Citation: González S, Oh D, Baclagon ER, Zheng JJ, Deng SX. Wnt signaling is required for the maintenance of human limbal stem/progenitor cells in vitro. *Invest Ophthalmol Vis Sci*. 2019;60:107–112. <https://doi.org/10.1167/iovs.18-25740>

PURPOSE. A chemical approach to examine the role of Wnt signaling in maintaining the stemness and/or proliferation of limbal stem/progenitor cells (LSCs).

METHODS. LSCs were isolated from human donor eyes and cultured as single cells for 12 to 14 days with the following small molecules: IIC3, an antagonist of the Wnt signaling inhibitor Dickkopf (DKK), and IC15, a Wnt signaling inhibitor. Proliferation of LSCs in the presence of IIC3 and IC15 was determined by the number of cells and colonies established. Maintenance of stemness was determined by p63 α , cytokeratin (K)12, and K14 expression.

RESULTS. Activation of Wnt, through IIC3-mediated DKK inhibition, resulted in similar colony forming efficiency (CFE) as in the untreated LSCs, but significantly increased the number of cultivated cells 7.21% with 5 μ M. Inhibition of Wnt with IC15 significantly reduced the CFE ($P \leq 0.01$) and the number of cultivated cells by 16% to 29%. Percentage of cells expressing high levels of p63 α (p63 α^{bright}) and quantity of small cells ($\leq 12 \mu\text{m}$), which contain the LSCs, increased 4.71% and 11.26% (both $P < 0.05$), respectively, with 5 μ M IIC3. All concentrations of IIC3 and IC15 retained the K14 undifferentiated marker (97%), while differentiation, as detected by expression of K12, was found in up to 2% of cells in 1 μ M IIC3, 1 μ M IC15, or 5 μ M IIC3.

CONCLUSIONS. Wnt signaling is required in LSC proliferation and maintenance of an undifferentiated state. The current study is a proof of concept that the Wnt pathway could be modulated in LSCs to enhance or decrease the efficiency of human LSC expansion.

Keywords: limbal stem cells, Wnt, small molecules

Proper Wnt signaling is critical to regulate numerous processes including cellular polarity, multiplication of cells, and cellular differentiation.^{1–3} These processes are initiated by one of three major signaling cascades that are activated once secreted Wnt ligands bind their Frizzled (FZD) transmembrane receptors.^{4,5} The most well-studied pathway in Wnt signaling is the canonical Wnt/ β -catenin pathway. Canonical signaling prevents the degradation of β -catenin when Wnt binds to FZD and its coreceptor LRP5/6. This allows β -catenin to translocate into the nucleus to transcriptionally activate genes.^{1,6,7} Other signals mediated by Wnt-FZD binding are the noncanonical pathways, including the Wnt/c-Jun N-terminal Kinase (JNK) pathway, and the Wnt/Ca²⁺ pathway, which are both β -catenin independent and are involved in regulating cellular polarity and migration.^{4,8}

Because many cellular processes are controlled through Wnt activation, it is not surprising that Wnts are involved in modulating stem cells that go through periods of quiescence, proliferation, and differentiation. The expression of Wnt has been found in a variety of cell types including embryonic,^{9–11} hair follicle,^{12,13} and intestinal stem cells.^{14,15} Furthermore, additional stem cell populations regulated by the Wnt pathway are continuously being discovered, especially in replenishing tissues, such as the cornea. The cornea is the very first structure that light encounters as it enters the eyes, and the epithelium is constantly regenerated.^{16–19} The corneal epithelium must be regenerated because the epithelial cells are constantly shed as we blink and rub our eyes to maintain hydration and remove

debris. The inability to replenish corneal epithelial cells results in the deterioration and/or loss of vision.^{20,21} Stem cells for the corneal epithelium have been found in the limbal epithelium and are thus identified as limbal stem/progenitor cells (LSCs).^{22–24} We have previously shown that Wnt signaling was present in the LSC niche, and specific Wnt molecules and inhibitors were differentially expressed between the limbus and cornea.^{25,26} Subsequently, studies have supported the importance of Wnt in the regulation of LSCs by demonstrating increased proliferation upon in vitro activation of Wnt.^{25–28}

The canonical Wnt signaling cascade is initiated once Wnt establishes an interaction with the FZD and LRP5/6 coreceptors. This signaling is regulated by several Wnt inhibitors; one of those is Dickkopf (DKK),²⁹ as it blocks Wnt signaling by binding to LRP5/6. Studies by Li et al.³⁰ showed that DKK can be effectively inhibited by a small molecule, IIC3 (Gallocyanine); thus IIC3 activates Wnt signaling.²⁹ On the other hand, another small molecule, IC15, binds to the Wnt binding site of LRP5/6 and inhibits Wnt signaling.²⁹ Therefore, pairing IIC3 and IC15 together makes a perfect tool to probe canonical Wnt signaling in biological systems.

Previously, studies have only targeted factors after Wnt receptor binding, and until now small-molecule studies on Wnt receptors have not been performed on limbal epithelial cells (LECs). Here, using the two small molecules, we demonstrate the effect of activating and prohibiting Wnt signaling on human primary LECs in vitro. By activating Wnt with IIC3 or preventing Wnt activation by blocking Wnt-LRP5/6 interactions

with IC15,^{30,31} the current study shows that Wnt is a key component in the preservation and proliferation of LSCs in culture.

MATERIALS AND METHODS

Human Corneoscleral Tissue

Human LSC cultures were derived from three sclerocorneal tissue donors (ages ≤ 75 years). All tissues were preserved in Optisol (Chiron Ophthalmics, Inc., Irvine, CA, USA) and obtained from CORE eye bank, Sightlife, and Lions eye bank. The death-to-preservation time in Optisol was less than 10 hours, and the time from death to experiment was 4 to 6 days. Experimental protocols were evaluated by the University of California, Los Angeles Institutional Review Boards and performed in accordance with the Declaration of Helsinki.

Isolation and Cultivation of Limbal Epithelial Cells

Isolation and cultivation of LSCs were performed as previously described.³² Briefly, LEC sheets were isolated from corneoscleral rims following incubation with 2.4 U/mL Dispase II (Roche, Indianapolis, IN, USA) in SHEM growth medium (DMEM/F12; Gibco, Carlsbad, CA, USA) supplemented with N-2 (Gibco), 2 ng/mL epidermal growth factor (EGF; Gibco), 8.4 ng/mL cholera toxin (Sigma-Aldrich Corp., St. Louis, MO, USA), 0.5 $\mu\text{g/mL}$ hydrocortisone (Sigma-Aldrich Corp.), 0.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich Corp.), and 5% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) for 2 hours at 37°C. LEC sheets were gently lifted from the tissues and single cells were obtained by treatment with 0.25% trypsin-EDTA (Gibco) at 37°C for 5 minutes. The final concentration of DMSO was adjusted for all cultures to be 0.5% (vol/vol). The LEC single cells were seeded at 200 cells/cm² on mitomycin-treated 3T3 cells (3×10^4 3T3 cells/cm², from Howard Green, Harvard Medical School, Boston, MA, USA) in SHEM medium with the same final DMSO concentrations (0.5%). Each experiment used LSCs at passage 0.

To investigate the effect of the Wnt pathway on LSCs, IIC3 (Sigma-Aldrich Corp.) or IC15 (Sigma-Aldrich Corp.) was added to the culture medium with final concentrations of 1, 5, 10, or 20 μM . Cultures without small molecules served as the control. Cell culture medium was routinely changed every 2 to 3 days containing the appropriate concentration of small molecules until collection between 12 to 14 days. The cultured LECs were then characterized.

Cell population doubling from all the conditions tested was calculated by using the formula Log_2 (no. cells harvested/no. cells seeded).

The short-term effect of IIC3 and IC15 treatment was also tested on freshly isolated LECs after 5-, 10- and 30-minute incubations with the most efficient (5 μM) and highest (20 μM) concentration of small molecules. After incubation, the cells were stained with trypan blue to determine cell viability.

Colony Forming Efficiency

Cells were fixed with 4% paraformaldehyde (ThermoFisher Scientific, Carlsbad, CA, USA) and stained for 15 minutes with 0.5% rhodamine B (Sigma-Aldrich Corp.) at room temperature. The number of cells plated was then divided by the number of colonies formed to obtain the CFE.

Immunocytochemistry

Colonies were harvested with dispase (2.5 mg/mL; Roche) and treated with trypsin-EDTA (0.25%; Gibco) to isolate single cells.

Single cells were counted using a hemocytometer and washed in SHEM growth medium before setting onto slides with a cytospin (Cytospin; Fisher Scientific, Houston, TX, USA). Cells were fixed with 4% paraformaldehyde at 20°C for 10 minutes, washed with phosphate-buffered saline (PBS), incubated with 1% bovine serum albumin (BSA) and 0.5% Triton X-100 (Sigma-Aldrich Corp.) in PBS at 20°C for 30 minutes, and incubated with the following primary antibodies at 4°C overnight: K12 (Santa Cruz Biotechnology, Dallas, TX, USA; sc-25722, 1:100), K14 (Fisher Scientific; MS-115-R7, 1:50), p63 α (Cell Signaling Technology, Danvers, MA, USA; 4892, 1:100), Ki67 (DAKO, Carpinteria, CA, USA; M7240, 1:100). Cells were washed with PBS, then incubated with the secondary antibodies goat anti-mouse IgG, Alexa Fluor 488 (ThermoFisher Scientific, A11029, 1:500), goat anti-rabbit IgG, Alexa Fluor 546 (ThermoFisher Scientific, A11035, 1:500) at 20°C for 1 hour. After washing with PBS, nuclei were labeled with Hoechst 33342 (4 $\mu\text{g/mL}$; Invitrogen) at 20°C for 15 minutes and mounted in Fluoromount medium (Sigma-Aldrich Corp.).

Images were taken with the digital inverted Keyence BZ-X710 fluorescence microscope (Osaka, Japan) and the image capture system BZ-X Viewer. Quantification of marker expression was performed by using the BZ-X analyzer software (version 1.3.0.3) and the hybrid cell count function. By specifying a mask area, the software extracts information on multiple parameters such as intensity of fluorescence signal in different channels, cell counts, and target area measurements. Cells expressing high levels of p63 α (p63 α^{bright} cells) were also quantified following this method as previously described.³³

Statistical Analysis

Data obtained were analyzed using Student's *t*-test, and error bars represent calculations obtained from standard error of the mean (SEM). Significance was determined at *P* values ≤ 0.05 .

RESULTS

Morphology and Proliferation of LSCs Cultured With IIC3 and IC15 Small Molecules

The IIC3 and IC15 small molecules were identified to bind YWTD repeat domains of LRP5/6, which included sites for DKK and Wnt binding.³⁰ Studies have also shown IIC3 to compete effectively against DKK at 5 μM , promoting Wnt activation. Alternatively, IC15 interfered with Wnt-LRP5/6 binding, and was found to inhibit Wnt signaling at concentrations above 1 μM .³⁰ To investigate the effect of the Wnt pathway on LECs, single cells isolated from LEC sheets were cultured with varying concentrations (1, 5, 10, 20 μM) of IIC3 and IC15.

Morphologic evaluation of LECs cultured with either small molecule showed similar cuboidal appearances to untreated control cells at all concentrations (Fig. 1A). Colony forming efficiency (CFE), an indicator of the amount of progenitor/stem cells, was also evaluated. The limbal epithelium is known to have a small population of stem/progenitor cells, typically less than 10%.³⁴ Accordingly, untreated cells had an average CFE of 8.76%; however, treatment with the IC15 inhibitor resulted in significantly reduced CFE for all tested concentrations (Figs. 1B, 1C). Inhibition of DKK with IIC3 did not increase CFE compared to the control. CFE was significantly reduced upon exposure to the highest concentration of activator, 20 μM IIC3. In parallel, cell population doubling displayed a similar pattern in which a decrease was detected with increasing concentrations of IC15 with significance at 10 and 20 μM , while population doubling was relatively similar at all

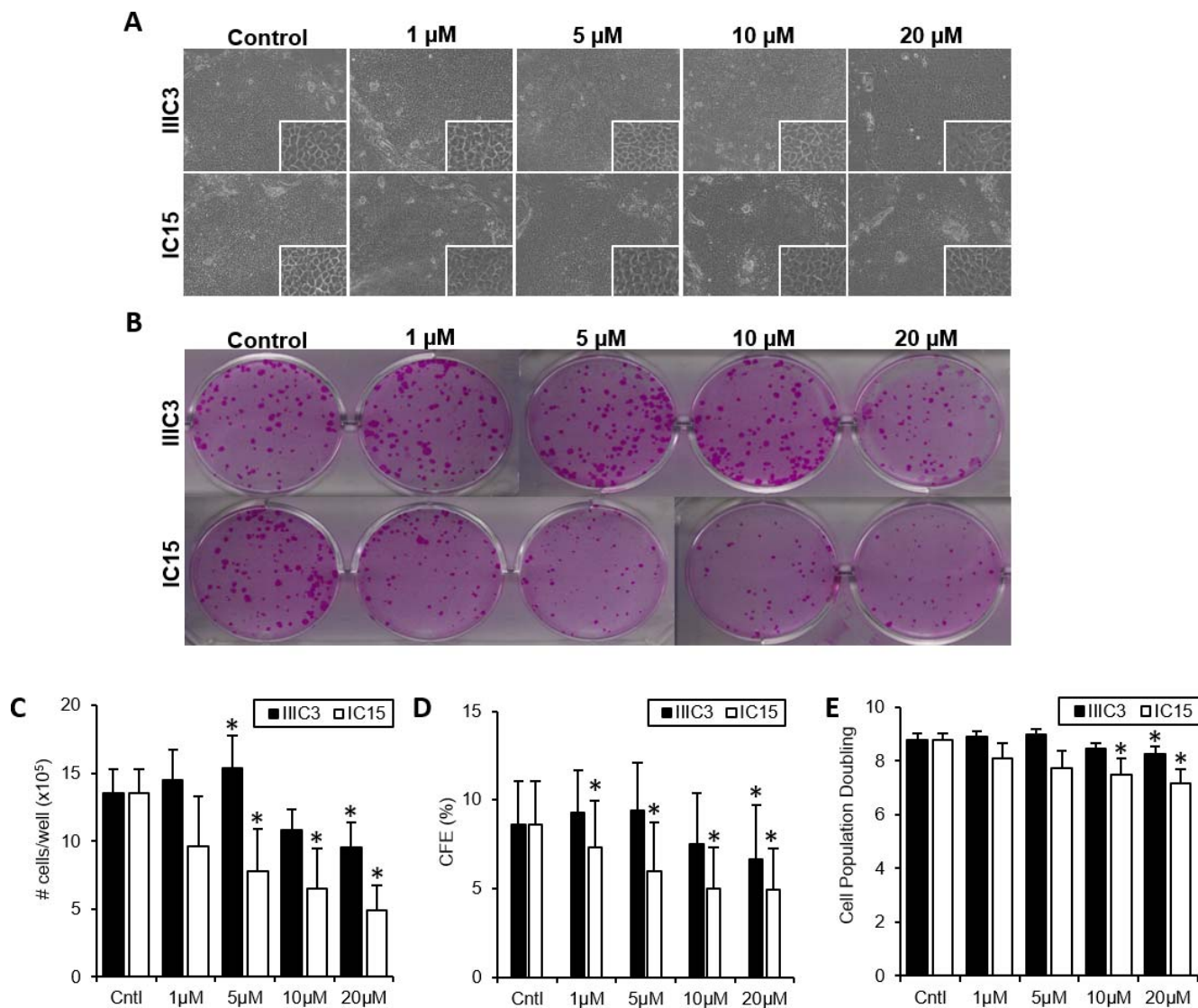


FIGURE 1. Effect of IIC3 and IC15 on limbal stem/progenitor cell (LSC) proliferation. (A) Treatment of LSCs with IIC3 and IC15 did not change the cuboidal morphology. (B–E) Different concentrations of IIC3 and IC15 affect colony forming efficiency (CFE) and cell proliferation of LSCs. IIC3 treatment generated larger colonies while IC15 treatment generated colonies of smaller size (B). The CFEs obtained from IIC3 treatment were similar to those for untreated controls except at the highest concentration of 20 μ M. CFE was significantly reduced with all concentrations of IC15 (C). Cell population doubling calculated as Log_2 (no. cells harvested/no. cells seeded) (D). Cell numbers were significantly increased with 5 μ M IIC3, but decreased in a dose-dependent manner for IC15 treatment, and 20 μ M IIC3 (E). * $P < 0.05$.

concentrations apart from a significant decrease at 20 μ M (Fig. 1D). As for the number of cells grown in culture, treatment with IC15 gradually reduced the number of cultivated LECs (16%–29%), while 5 μ M IIC3 increased cell number by 7.21% and 20 μ M IIC3 decreased it by 29.4% (Fig. 1E).

To determine if these differences were due to changes in the number of cells entering the cell cycle after small-molecule treatment, we assessed the cells for Ki67, which is highly expressed during the cell cycle but absent in quiescent cells.³⁵ Calculations of Ki67⁺ percentages between cultures showed no significant differences between all tested concentrations of IIC3 and IC15 treatment (Fig. 2B).

To investigate whether the small molecules have direct cytotoxic effects on LECs, IIC3 and IC15 were incubated with freshly isolated LECs for 5, 10, and 30 minutes. The cell viability was similar between control (92.2%–94%), IIC3 (88.9%–98.1%), and IC15 (94.7%–96.1%) small-molecule groups at 5 μ M ($P < 0.05$), while at 20 μ M, the percentage

of trypan blue–stained cells increased for both IIC3 (12.8%) and IC15 (7.6%) at 10 minutes but remained stable at 30 minutes compared to that of the control. This observation suggests that the effects at high concentrations were likely due to the inhibition of Wnt signaling alone.

Maintenance of LSC Phenotype After Small-Molecule Treatment

LSCs are distinguished from differentiated corneal cells through p63 α expression.^{36,37} In order to confirm that the colonies contained specifically LSCs, the number of cells expressing high levels of p63 α (p63 α^{bright} cells) was quantified in the cultivated LEC population. The percentage of p63 α^{bright} cells significantly increased by 4.71% with 5 μ M IIC3 activation ($P = 0.04$, Figs. 3A, 3B), but inhibition of Wnt signaling with IC15 resulted in a significant loss of p63 α^{bright} cells. Even with the lowest dose of 1 μ M IC15 there was a

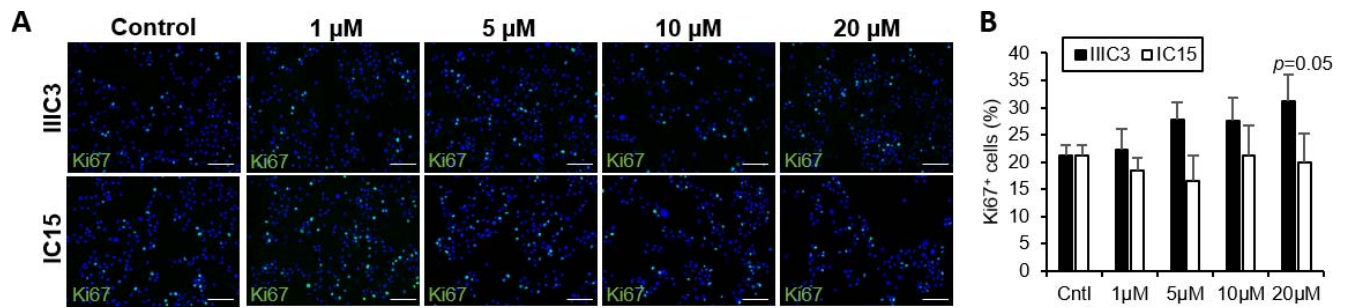


FIGURE 2. LSCs continue to actively divide upon small-molecule treatment. (A) Immunostaining of Ki67 in LSCs treated with IIC3 and IC15. (B) Ki67 expression is significantly increased in LSCs treated with 20 μM IIC3. Percentage of Ki67⁺ cells showed no significant differences between all tested concentrations of IIC3 and IC15. Scale bar: 20 μm. * $P < 0.05$.

decrease of 2.91% p63^α^{bright} cells ($P = 0.04$), while at the highest concentration of 20 μM IC15 a 9.08% ($P = 0.03$) decrease was observed in addition to the significantly reduced total p63^α^{bright} cell population (>85,000 cells; $P < 0.05$, Fig. 3C). Furthermore, cell size was evaluated as LSCs are typically quiescent with reduced cytoplasmic content, resulting in higher nuclear-cytoplasmic ratios and sizes smaller than 12 μm.^{38–40} Analyses revealed that the percentage of small cuboidal cells was significantly increased only with 5 μM of IIC3 (Fig. 3D).

The LSCs are further characterized by the expression of the cytokeratin marker K14, while differentiation is marked by the expression of K12. Roughly 97% of LECs treated with either IIC3 or IC15 retained the K14 marker, and the K12 differentiation marker was only significantly increased with 1 μM IIC3, 1 μM IC15, and 5 μM IIC3 (Fig. 4). As the differentiation was detected in only approximately 2% of cells, it appears that most LSCs remain in an undifferentiated state in culture.

DISCUSSION

From the maintenance of stem/progenitor characteristics to promoting the multiplication of stem cells, many previous studies have examined the Wnt pathway to determine how cellular processes are regulated. Specifically, factors downstream of the Wnt-FZD interaction were targeted to either prevent or activate the transcription of genes. Recently, however, modifications to the primary components, such as the FZD and LRP5/6 receptors, or associated inhibitors, such as DKK, have also been demonstrated to have significant effects on cellular responses.^{30,41} As these previous studies demonstrated the potency of inhibitors and receptors on the Wnt pathway we decided to investigate how modulation of primary Wnt components using small molecules would affect LSCs in vitro. Our results show that inhibition of the Wnt inhibitor DKK using IIC3 enhances the proliferation of LSCs in a dose-dependent manner, while inhibition of Wnt signaling using IC15 reduces the stem cell proliferation and quantity.

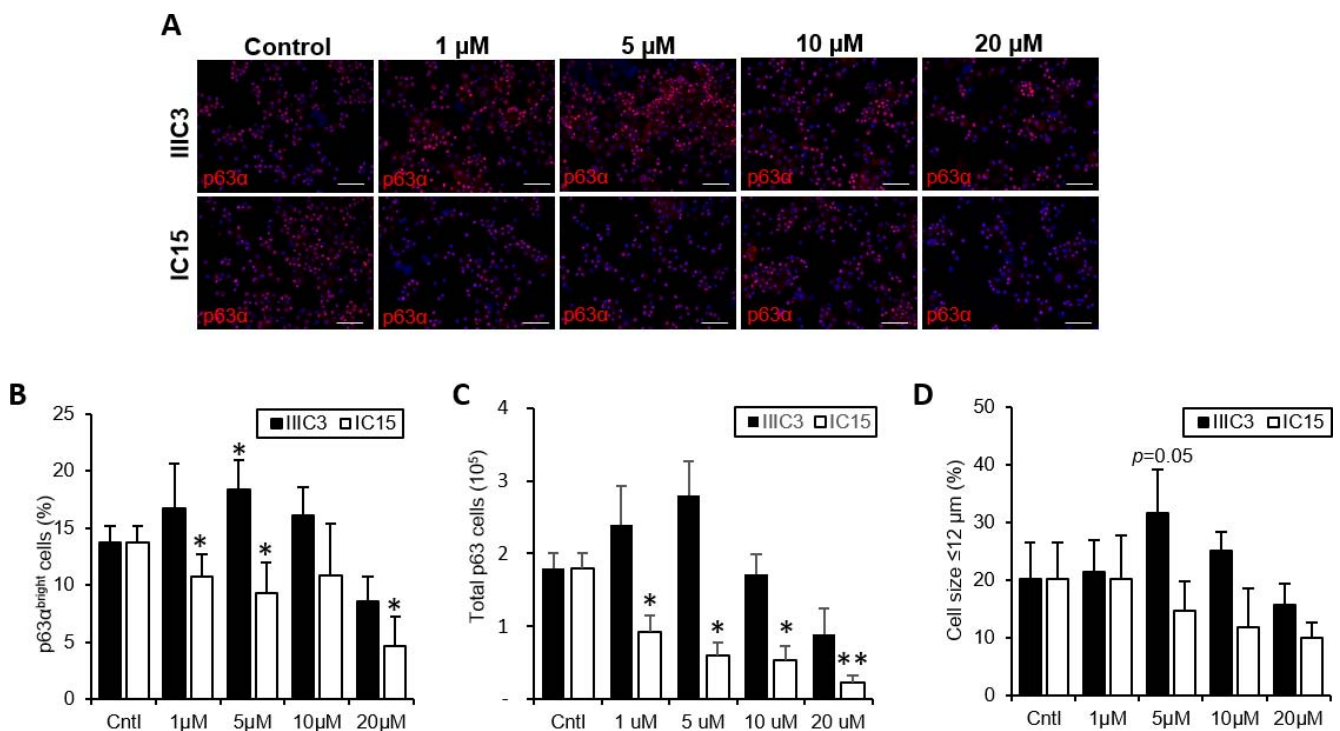


FIGURE 3. Maintenance of limbal stem/progenitor cell (LSC) undifferentiated phenotype is IIC3 concentration dependent. (A) Expression of p63^α is retained in LSCs upon IIC3 treatment but reduced with all tested concentrations of IC15. (B, C) Quantification of p63^α^{bright} cells showed a significant increase with 5 μM IIC3. IC15 treatment reduced the percentage and the total number of p63^α^{bright} cells (D). The percentage of cells maintaining a cell size smaller than 12 μm was significantly increased with 5 μM IIC3. There was a trend of decrease with IC15 treatment. * $P < 0.05$, ** $P < 0.001$.

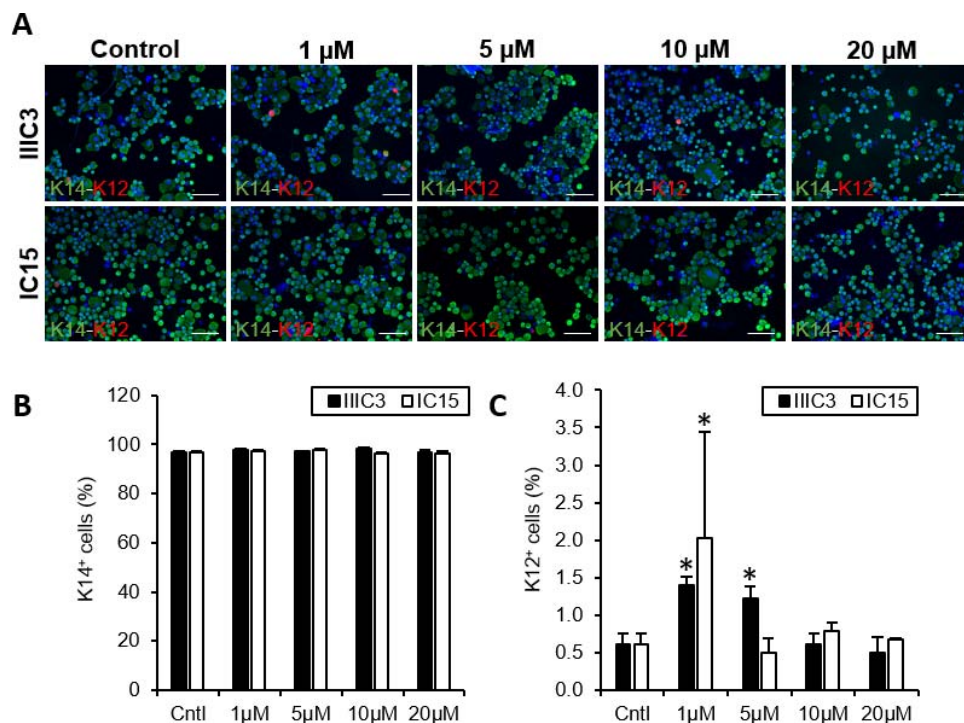


FIGURE 4. Expression of K14 is maintained by LSCs treated with IIC3 or IC15. (A) Immunostaining of K14, the LSC marker, and K12, the differentiation marker. (B) Most cells retain K14 expression. (C) K12 is detected in a small population of LSCs, and is increased within a narrow range, 1 to 5 μ M IIC3 and 1 μ M IC15. * $P < 0.05$.

The concentration dependency that we have observed is most likely due to the binding locations of IIC3. The extracellular domain of Wnt coreceptor LRP5/6 has four YWTD domains.^{30,41} Although IIC3 preferably binds to the second YWTD repeat domain of LRP5/6 where DKK binds, the similarity among the four YWTD repeat domains of LRP5/6 may also result in IIC3 binding to the third and fourth YWTD repeat domains of LRP5/6 at higher concentrations and block the Wnt binding sites.^{30,41} As a result IIC3 may behave as an inhibitor at higher concentrations. Additionally, as previous studies have found 5 μ M of IIC3 to be the most effective concentration to activate canonical Wnt signaling,³⁰ it appears that this concentration also efficiently improves proliferation of LSCs demonstrated by our 7.21% increase. However, 5 μ M IIC3 did not increase the CFE. This is not surprising as colony formation is limited by the number of LSCs seeded, which is only 10% of the limbus, at maximum. The CFE is further limited by the cell's potential to establish a colony, leave the quiescent stem state, and promote cellular division. These cells must also continue to maintain LSC characteristics during these proliferative states, such as small cell size and the expression of high levels of p63 α . Both of these were significantly increased upon treatment with 5 μ M IIC3. Inhibition with IC15, however, significantly decreased the number of p63 α ^{bright} cells to a much larger extent as the total number of p63 α ^{bright} cells decreased by more than 85,000 cells with a small percentage being p63 α ^{bright} positive. At high IIC3 concentrations, the LSC population did not increase, and appeared to have inhibitory effects as the number of cultured cells was reduced. This is expected because of the inhibitory binding sites of IIC3 on LRP5/6 at high concentrations. As inhibition of Wnt signaling with IC15 significantly reduced cell proliferation and CFE, our results suggest that Wnt activity is present in the limbus and that it is required for LSC maintenance.

Short-term incubation of the LECs with 20 μ M IIC3 and IC15 showed a slight reduction in cell viability but remained

stable after 10 minutes, whereas at 5 μ M both small molecules did not show differences in cell viability compared to the control. If these small molecules did have direct cytotoxic effects, the viability would be expected to decrease in a linear fashion. The fact that the viability remained stable after 10 minutes suggests that the initial effect is likely due to inhibition of Wnt signaling at high concentrations (20 μ M), and implies that Wnt signaling may be involved in LEC survival pathways, warranting further investigation.

We previously found that Wnt 2, 6, 11, and 16b were preferentially expressed in the limbus along with four Wnt inhibitors DKK1, Wnt inhibitory factor 1, FRZB, and secreted frizzled-related protein 5.²⁶ We hypothesize that Wnt signaling is balanced by Wnt inhibitors during quiescent states, and these inhibitors must be modulated carefully to promote the proliferation and differentiation of LSCs. However, exactly how the Wnt pathway is regulated remains elusive due to the number and complexity of components and their roles in signaling.

In this study we conclude that the Wnt pathway is required in LSC proliferation and the maintenance of undifferentiated LSCs. Careful regulation of the Wnt pathway is critical as the levels can promote or decrease the efficiency of human LSC expansion. Further small-molecule studies on Wnt and their activities in LSC proliferation and differentiation are needed as they could have translational potential in maintaining a functional corneal epithelium, a major contributor to vision.

Acknowledgments

Supported by the National Eye Institute (R01EY021797 and 5P30EY000331), California Institute for Regenerative Medicine (TR2-01768, BF1-01768), and an unrestricted grant from Research to Prevent Blindness.

Disclosure: S. González, None; D. Oh, None; E.R. Baclagon, None; J.J. Zheng, None; S.X. Deng, None

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