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Frizzled 7 Maintains the Undifferentiated State of Human Limbal Stem/Progenitor Cells

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Abstract

Wnt signaling pathway plays an important role in the regulation of human limbal stem/progenitor cells (LSCs). To examine the possible function of Frizzled (Fz) receptors in LSCs, the expression of ten Fz receptors was profiled in the limbus and cornea. Only Fz7 had preferential expression in the basal limbal epithelium which contains the LSCs. The expression of Fz7 was co-localized with the putative LSC markers including p63α, N-cadherin and keratin (K) 14 and was minimum in cells expressing the corneal maturation marker K12. The expression of Fz7 was higher in the enriched LSCs population and decreased in cultured LSCs when there was a loss of progenitor phenotype. When the Fz7 was knocked down (Fz^{KD}) using shRNA in primary LSCs, the expression of putative LSCs markers ABCG2, ΔNp63α and K14 was decreased significantly. The colony forming efficiency of the $Fz7^{KD}$ LSCs was significantly decreased in the subsequent passage 1 and 2 compared to the control. Our finding suggests that Wnt signaling is one of the factors of LSC niche maintenance and Fz7 helps to maintain the undifferentiated state of LSCs.

Keywords

limbal stem cells; Frizzled; niche factor; limbal stem cell deficiency; Wnt signaling pathway

Introduction

Corneal epithelial stem/progenitor cells (limbal stem cells, LSCs) are found at the limbus in human [1–4]. Efforts are made to identify the signaling pathways related to LSC proliferation and differentiation. Some conventional signaling pathways regulating cell proliferation and differentiation have been reported to be the potential regulators due to their preferential expression in the more differentiated corneal epithelium or in the less differentiated limbal epithelium, including c/EBP, Wnt, Notch and TGF-β pathways [5–10].

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In the Wnt signaling pathway, binding of secreted Wnt ligands to membrane-bound Frizzled (Fz) receptors and co-receptors, activates the intracellular signaling including β-catenin-dependent canonical pathway and β-catenin-independent non-canonical pathway. Wnt signaling pathway has been suggested to be an important niche factor in varies types of stem cells regulating their proliferation and differentiation. In hematopoietic stem cells (HSC), Wnt ligands are produced by both HSC and HSC niche cells [11, 12]. Activation of Wnt signaling promotes the proliferation of HSC and benefits their stem cell phenotypes [13]. In intestinal epithelial stem cells, Wnt ligands are secreted by the myofibroblast niche cells [14, 15] and inhibition of Wnt signaling in mice reduces intestinal epithelial proliferation and loss of crypts [16]. In mammary epithelial cells, Wnt signaling increases cell proliferation and promotes tumorigenesis [17–20]. In epidermal stem cells, activation of Wnt signaling leads to increased cell growth of hair follicles [21, 22]; disrupted Wnt signaling leads to the absence of hair and blocks the differentiation of stem cells into hair follicle keratinocytes, instead the differentiation into epidermal keratinocytes is adopted [23].

Wnt signaling may regulate limbal epithelial stem cell self-renewal and differentiation. It has been reported that Wnt4 is up-regulated in fetal limbal cornea compared to fetal central cornea and in primary cultured limbal epithelium compared to the cells after 5 passages [24]. Our previous studies revealed several Wnt signaling components preferentially expressed in the limbus than in the cornea in both adult monkey and human [9, 25]. In addition, activation of Wnt/β-catenin signaling using LiCl increases the proliferation and colony-forming efficiency (CFE) of cultured human limbal epithelial cells [8], suggesting an active role of Wnt signaling in LSCs regulation. However, the exact molecule(s) which exerts the regulating effect is largely unknown. The current study focuses on the Fz receptors of Wnt signaling and examines their expression patterns and possible function in regulating human LSCs.

Methods

Human sclerocorneal tissue

Human sclerocorneal tissue was obtained from the Illinois Eye Bank (Watson Gailey, Bloomington, IL) and the Lions Eye Institute for Transplant and Research (Tampa, FL). Tissue donors ranged in age from 20 to 65 years. Experimentation on human tissue adhered to the tenets of the Declaration of Helsinki. The experimental protocol was evaluated and exempted by the University of California, Los Angeles Institutional Review Boards.

The tissues were preserved in Optisol (Chiron Ophthalmics, Inc., Irvine, CA), and the death-to-preservation time was less than 10 hours.

Differential profiling of Fz receptor in human cornea and limbus

As described previously [25], human corneal and limbal epithelium together with its adjacent anterior stroma was dissected out from the sclerolimbal rim. RNAs were extracted (RNeasy Mini Kit, Qiagen, Valencia, CA), Dnase-treated (DNA-free kit, Ambion, Austin, TX) and reverse-transcribed into cDNA (SuperScript II, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quantity and quality of RNA was examined by Nanodrop (Thermo Scientific, Wilmington, DE). Transcripts were detected using Kapa Sybr Fast qPCR kit (Kapa Biosystems, Woburn, MA). The cycle conditions are as follows: the reactant was denatured for 20s at 95°C, amplified for 40 cycles: 95°C for 3s; 60°C for 20s; 72°C for 8s, then went through the dissociation curve. The primers used in qRT-PCR were listed in Table 1.

Human cornea was cryosectioned at a thickness of 8μm, mounted onto slides and stored in −80°C until used. Sections were fixed with 1% paraformaldehyde for p63α or 4%

paraformaldehyde for other markers on ice for 15 min and washed with PBS containing 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 3 times. The tissue was blocked with 10% donkey or goat serum in phosphate buffered saline (PBS) for 30min at room temperature followed by incubation with primary antibody diluted in 1%BSA in PBS for overnight at 4°C in a moisture chamber. Sections were washed 3 times with 0.3% Triton X-100 in PBS, and then incubated with corresponding secondary antibody at room temperature for 1h. After three washes with 1%BSA/0.3% Triton X-100 in PBS, the tissue was stained for nuclei with 4μg/ml Hoechst 33342 (Invitrogen) at room temperature for 15 min followed by 5 washes with PBS. The slides were mounted in Fluoromount mounting medium (Sigma-Aldrich). The primary and secondary antibodies used and their dilution factors are listed in Table 2.

Human LSC culture

Limbal epithelial cells were isolated from corneoscleral rims leftover from corneal transplantation surgery as previously described [26]. In brief, the residual blood vessels, iris, endothelium, Tenon's capsules and conjunctiva were removed. The rim was incubated in 2.4U/ml Dispase II (Roche, Indianapolis, IN) in LSC growth medium that was consisted of DMEM/F12 medium (Gibco, Grand Island, NY) supplemented with N2 (Gibco), 2ng/ml EGF (Gibco), 8.4ng/ml cholera toxin (Sigma-Aldrich), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 0.5% DMSO (Sigma-Aldrich), 5% FBS (Invitrogen), penicillin-streptomycin (Invitrogen) and gentamicin/amphotericin B (Invitrogen) for 2 hours at 37°C. The limbal epithelium was removed. Some cells were cytospined onto slides for immunocytochemistry and the rest of the cells were further trypsinized into single-cell suspension by incubating the cells in 0.25% trypsin/1mM EDTA (Gibco) for 10–15 min at 37°C.

Sub-confluent murine 3T3-J2 feeder cells were treated with 4μg/ml of mitomycin-C (Sigma-Aldrich, MO) for 2 hours at 37°C, trypsinized and sub-cultured in Dulbecco's modified Eagle's medium (DMEM) (ATCC, VA) supplemented with 10% FBS at a density of 3×10^4 cells/cm^2. Single limbal epithelial cell was seeded at a density of 300 cells/cm^2 on the feeder cells and cultured in the LSC growth medium for 2–3 weeks.

Enrichment of LSCs

Freshly isolated LSCs were segregated into stage-specific embryonic antigen-4 $(SSEA4)^+$ and $SSEA4^-$ cells based on their cell surface expression of SSEA4 using Dynabeads (Invitrogen) as described [27]. Briefly, single limbal epithelial cells were incubated with anti-SSEA4 antibody at room temperature for 15min and excess antibody was washed away. Secondary antibody linked to magnetic beads was added to the limbal epithelial cells. The $SSEA4^+$ cells were pulled down in a magnetic field. The cells in the suspension were $SSEA4^-$ population. The cells before sorting served as the control.

Knock-down of Fz7

The Fz7 shRNA-GFP construct, scrambled control–GFP construct and the packaging plasmids were generous gifts from Prof. Markus Müschen (Department of Laboratory Medicine, UCSF) [28]. Lentivirus was produced following Prof. Müschen's protocol [28]. In brief, 293T cells were seeded at 4×10^6cells/10cm dish and transfected with the virus containing the plasmid using polyethylenimine (PEI) on day2 (total DNA:PEI=1:2 in μg) for 24 hours. On day3 fresh LSC growth medium was added onto the cells (5ml medium/10cm dish) incubating for 24 hours. On day 4, the 1st batch of medium containing the virus were collected and fresh LSC growth medium was added. After 24 hours the 2nd batch of medium containing the virus was collected. The 1st and 2nd batches was pooled, aliquoted and stored in −80°C until use.

Freshly isolated human limbal epithelial cells were transduced by mixing 20,000 cells with 4 ml virus-containing medium (4ml/10cm dish) and 2.5μg/ml polybrene. The mixture was incubated at room temperature for 15 min and added onto the growtharrested 3T3-J2 cells. After 24–36 hours incubation, the LSC growth medium was changed. The GFP-positive transduced cells were collected either by flow-cytometry or by scraping away the non-GFP colonies.

The GFP-positive cells were lysed for RNA extraction (RNeasy Micro Kit, Qiagen, Valencia, CA) to examine the expression profile following the same protocol of reverse-transcription and qRT-PCR as mentioned above. At the step that the cell lysates went through the MinElute columns, the nucleic acids were retained on the membrane and the proteins were eluted out. Proteins were precipitated from the eluent by acetone following the Appendix F of the manufacturer's instructions. 25 μg of total proteins were separated by one-dimentional NuPAGE 4–12% Bis-Tris gel (Invitrogen) and transferred to polyvinylidine difluoride (PVDF) membranes. The membranes were blocked by SuperBlock Blocking Buffers (Thermo Scientific) and incubated with primary Ab diluted in 1X Tris Buffered Saline with Tween-20 (TBS-T) containing 0.1% Tween-20 and 5% BSA at 4°C overnight. Membrane was washed with 1X TBS-T three times and incubated with HRP-conjugated secondary antibodies for 2h at room temperature. The antibodies used and the dilution factors are listed in Table 2.

Colony-forming efficiency (CFE)

The GFP-positive cells were seeded at 300 cells/cm^2 onto the 3T3-J2 feeder cells for 2 weeks to form colonies. The CFE was calculated by dividing the number of colonies by the number of cells seeded.

Statistical Analysis

Student's t-test was performed to analyze transcript levels and CFEs. $P \leq 0.05$ was considered statistically significant.

Results

Preferential expression of Fz receptors in the human cornea and limbus

The expressions of ten Fz receptors, Fz1 to Fz10 were examined by comparing their mRNA levels between cornea and limbus using qRT-PCR. Among the ten Fz receptors screened, four receptors Fz1, Fz4, Fz7 and Fz10 consistently had a significantly higher expression in the limbus and Fz8 had a higher expression in the cornea (Fig. 1A). The expression in the cornea was 87%, 82%, 87% and 50% lower, respectively for Fz1, Fz4, Fz7 and Fz10 than that in the limbus (all $p<0.05$). The expression of Fz8 was 42% lower in the limbus than that in the cornea ($p<0.05$). Their protein expression patterns were further examined by immunohistochemistry except Fz10 due to the lack of antibody (Fig. 1B). Although Fz1 had an overall higher expression in the limbal epithelium than in the corneal epithelium, it was expressed at all layers of the limbal and corneal epithelium. Fz4 was found at the basal layer of limbal epithelium. It was also strongly expressed at the suprabasal and superficial layer of the limbal epithelium and the superficial layer of the corneal epithelium Fz7 was strongly and preferentially expressed at the basal layer of limbal epithelium while its expression in the cornea was minimum. Fz7 appeared to be expressed at a higher level at the basal and lateral side than the apical side of the basal limbal epithelium. Fz7 was also strongly expressed in the immediately adjacent limbal stromal cells. Expression of Fz7 was most specific in the basal limbal epithelium where LSCs are located compared to Fz1 and 4. Therefore we focused our study on Fz7 in LSCs.

The Fz7 co-localized with putative LSC markers

The phenotype of the Fz7-expressing basal limbal epithelial cells was first investigated by their expression profile with several putative LSC markers including ΔNp63α, N-cadherin and K14, and the maturation marker K12. The p63α antibody recognizes all the isoforms; however ΔNp63α isoform is the most dominant isoform expressed in human limbal epithelium [29]. Therefore the expression of p63α could serve as an indicator of the expression of the putative stem cell marker ΔNp63α. p63α was expressed at all layers of limbal epithelium and clusters of p63α-bright cells were found at the basal limbal epithelium. Majority of the p63α-bright cells co-localized with Fz7-bright cells (Fig. 2, white arrow). However, there was a small population of $Fz7^{+}/p63\alpha^{-}$ (Fig. 2, white arrowhead) and $Fz7^{-}/p63\alpha^{+}$ basal limbal epithelial cells (Fig. 2, yellow arrow). N-cadherin was expressed specifically at the basal limbal epithelium as clusters. Many cells expressing N-cadherin also showed a high expression of Fz7 (Fig. 2, white arrow) but a small portion of N-cadherin^{+} cells had minimum Fz7 expression (Fig. 2 yellow arrow). Some $Fz7^{+}$ cells had low N-cadherin expression (Fig. 2, white arrowhead). K14 was highly expressed at the basal and suprabasal layers of limbal epithelium. Most K14-bright cells at the basal epithelium also had a high expression of Fz7 (Fig. 2 white arrow). K12, the corneal differentiation marker, was highly expressed in the central cornea and in the limbal suprabasal and superficial epithelial layers. At the segment of basal limbal epithelium where the Fz7 was highly expressed, the expression of K12 was absent (Fig. 2 white arrowhead). Colocalization of Fz7 expression with these markers was further confirmed by immunocytochemistry in the freshly isolated human limbal epithelial cells (supplementary Fig. 1A–D).

The expression of Fz7 was higher in the enriched LSC population and decreased in cultured LSCs when there was a loss of progenitor phenotype

We have previously showed that limbal epithelial cells contain two populations based on their surface expression of SSEA4 [27]. The $SSEA4^{-}$ limbal epithelial cells contained an enriched LSC population. The expression of Fz7 in the $SSEA4^{-}$ cells was examined (Figure 3A). $SSEA4^{-}$ cells had significantly higher expression of ABCG2 and K14 than the $SSEA4^{+}$cells, which is consistently with the previous finding. Interestingly, $SSEA4^{-}$ cells also had significantly higher expression of Fz7 than the $SSEA4^{+}$ population. This finding supports the above observation that Fz was preferentially expressed in stem/progenitor cell population.

When Fz7 expression was investigated in the LSC cultured on the growth arrested 3T3 cells, the limbal epithelial cells which contained the LSCs before and after culture were examined for their stem/progenitor cell phenotypes (Figure 3B). After culture, the limbal epithelial cells had significantly lower expression of the putative stem cell markers including ABCG2, ΔNp63 and N-cadherin. The expression of Fz7 was significantly reduced as well. This finding suggests a loss of stem cell phenotype after culture correlates with a loss of Fz7 expression. Because the culture using 3T3 feeder cells eliminates the differentiated $K12^{+}$ corneal epithelial cells and only expands the stem/progenitor population, the expression of K12 is expected to be significantly reduced after culture. The proliferation marker Ki67, was significantly increased after culture reflecting an active proliferating state of the cultured cells as expected.

Knockdown of the endogenous Fz7 expression leads to a loss of stem cell properties

To examine the function of Fz7 in LSCs, freshly isolated human limbal epithelial cells were transduced with lentivirus that contained plasmid construct of Fz7 shRNA-GFP or scrambled shRNA-GFP as control. The transfected cells were cultured on 3T3 feeder cells for 2–3 weeks to select for the LSC population (Passage 0, P0). The GFP-positive LSCs were selected and underwent phenotype analysis. Compared to the LSCs transduced with

control, the mRNA level of Fz7 was reduced by 37% in the Fz knocked-down (Fz^{KD}) cells transduced with Fz7 shRNA (Figure 4A). The reduced expression of Fz7 was further confirmed by Western blot at the protein level (Figure 4B). In the Fz^{KD} LSCs, the expressions of putative stem cell markers, K14, ABCG2, ΔNp63 were significantly reduced by 19%, 25% and 20%, respectively (Figure 4C). The expression of N-cadherin was reduced by 18%. The expression of the differentiation marker K12 in the Fz^{KD} LSC population was increased by 25%. Compared to the control group, the Fz^{KD} LSCs showed a significant decrease in the expression of proliferation marker Ki67, which indicates a lower proliferation rate (Figure 4C). The GFP-positive LSCs cells which contained either the Fz shRNA or control plasmid from P0 were passaged twice on 3T3 feeder cells to obtain P1 and P2 to examine the CFE (Figure 4D). The CFE in the Fz^{KD} LSCs was decreased significantly by 63% at P1 and decreased by 38% at P2 compared to the control group. This finding suggests that Fz7 might be necessary to maintain the stem/progenitor phenotype of LSCs.

The Fz7 expression co-localized with Syndecan-4 (Sdc4) and Fibronectin (FN) at the basal limbal epithelium

It has been reported that Fz7 forms a complex with Sdc4 and the binding of FN to this complex induces symmetric division of satellite stem cells stimulated by Wnt7a [30]. The expression of Fz7, Sdc4 and FN in human limbus was studied. The expression of Fz7 was co-localized with Sdc4 and FN in some cells at the basal layer of limbal epithelium, especially between some adjacent basal epithelial cells (Fig. 5 white arrow).

Discussion

Fz receptor expression was profiled in human cornea and limbus. Fz 1, 4, 7, and 10 had consistently higher mRNA expression in the limbus and Fz8 in the cornea. Localization of these Fz at the protein level shows that only Fz7 was preferentially expressed at the basal layer of limbal epithelium which suggests that it potentially could serve as a niche factor for LSCs and is the focus of the current study. The expression of high level of Fz7 showed minimum K12 expression, indicating that a high expression of Fz7 is indicative of undifferentiated status. Majority of the Fz7–bright cells also expressed high levels of p63α, N-cadherin, and K14. It is well known that the basal limbal epithelial cells are heterogeneous [31, 32]. They contain the stem/progenitor cells, the transient amplifying cells and niche cells [31, 32]. Our finding from the colocalization of Fz7 expression with other putative stem cell markers indicates that there are several populations of limbal basal epithelial cells: Fz7-bright/p63α-bright, $Fz7^{+}$/N-$cadherin^{+}$, $Fz7^{+}$/N-$cadherin^{-}$, $Fz7^{+}$/$K14^{+}$, and F7+/K12− cells. Although several molecules have been proposed to be biomarker of LSCs, none of them is specific. It is likely that more than one marker is necessary to define LSCs. The combination of these markers may suggest a stem/progenitor population. Therefore, Fz7 could serve as a potential LSC biomarker to further identify and enrich the stem/progenitor cell population.

The expression level of Fz7 correlates with the degree of stemness. Its expression level is significantly higher in the $SSEA4^{-}$ limbal epithelial cells, which contain the LSC-enriched population [27]. The expression of Fz7 was significantly reduced in the cultured limbal epithelial cells whose stem cell phenotypes significantly lost after culture. In addition, knocked down of Fz7 endogenous expression in LSCs leads to a loss of several putative LSC markers and a decrease in CFE. These results suggest that Fz7 also function to maintain the stemness of LSCs. Interestingly, the expression of Ki67, a proliferation maker, was reduced in the $Fz7^{KD}$ LSCs, indicating that Fz7 may also contribute to the proliferation of LSCs besides maintaining their stem cell phenotype.

Fz7 has been reported to regulate the stemness and proliferation of other stem cells. Fz7 is preferentially expressed in human embryonic stem cells and plays an important role in the self-renewal of embryonic stem cells [28]. In human mesenchymal stem cells (hMSCs), Fz7 promotes cell survival and chondrocyte formation, and suppresses osteogenesis [33]. However, the underlying mechanism of Fz7 in stem cells is largely unknown. In satellite stem cells, Fz7 forms a complex with Sdc4 and binding of FN to this complex enhances Wnt5a to induces the symmetric expansion [30]. The immunohistochemistry in human cornea showed that Fz7 co-localized with Sdc4 and FN in some cells locating at the basal layer of limbal epithelium, especially between adjacent basal epithelial cells. Whether Fz7 maintains the undifferentiated state and promotes proliferation ability of LSCs in vitro through a similar mechanism is yet to be elucidated.

Fz7 can transduce both the canonical and non-cannoical Wnt signaling pathways in cancer cells [34]. In a recent study by Zhang, et al. in Xenopus shows that Fzd7 signals via both the Wnt/β-catenin and Wnt/JNK pathways and that different thresholds of Wnt-Fzd7 activity coordinate progenitor cell fate, proliferation and morphogenesis [35]. Many of the Wnt signaling components including Frzb1, Lymphoid enhancer-binding factor-1 (LEF1) and Wnt2 are preferentially expressed in the limbal epithelial crypt where is thought to harbor LSCs [8, 24, 36]. Similar to Fz7, TCF4, a key transcription factor in Wnt canonical pathway, co-localizes with ABCG2 and p63 at the basal limbal epithelium [37, 38] and regulates LSC proliferation in vitro [38]. We have previously shown that Wnt 2. 6, 11 and 16b were preferentially expressed in the limbus [8]. Which of these Wnt ligands binds to Fz7 and the subsequent activation of the Wnt signal via the canonical or noncannonical signaling will need to be further investigated.

In summary, out data suggest that Fz7 is preferentially expressed in the LSCs and may serve as an additional marker to further identify LSCs. Fz7 might serve as a LSC niche factor to maintain the stem cell phenotype and regulate the proliferation of LSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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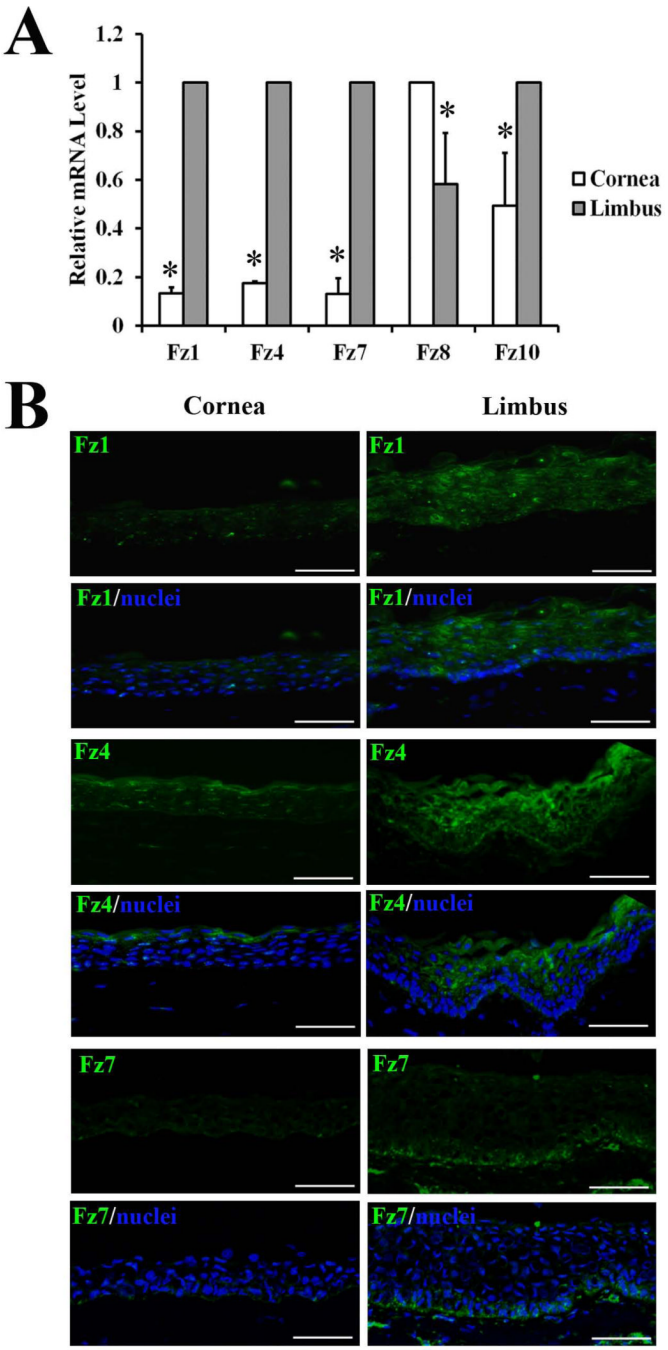

Figure 1.

Preferential expression of Fz receptors in human cornea and limbus. (A) The mRNA expression levels of Fz1, 4, 7, 8 and 10 in the limbus and cornea through qRT-PCR. Fz1, 4, 7 and 10 had significantly higher mRNA level in the limbus than in the cornea whereas Fz8 had a higher expression in the cornea. Error bar represents S.E.M. *:$p<0.05$. (B) Protein expression patterns of Fz1, 4 and 7 in human cornea and limbus by immunohistochemistry. Only Fz7 (green) was preferentially expressed at the basal limbal epithelium. The nuclei were co-stained with Hoechst (blue). Scale bar represents 50 μm.

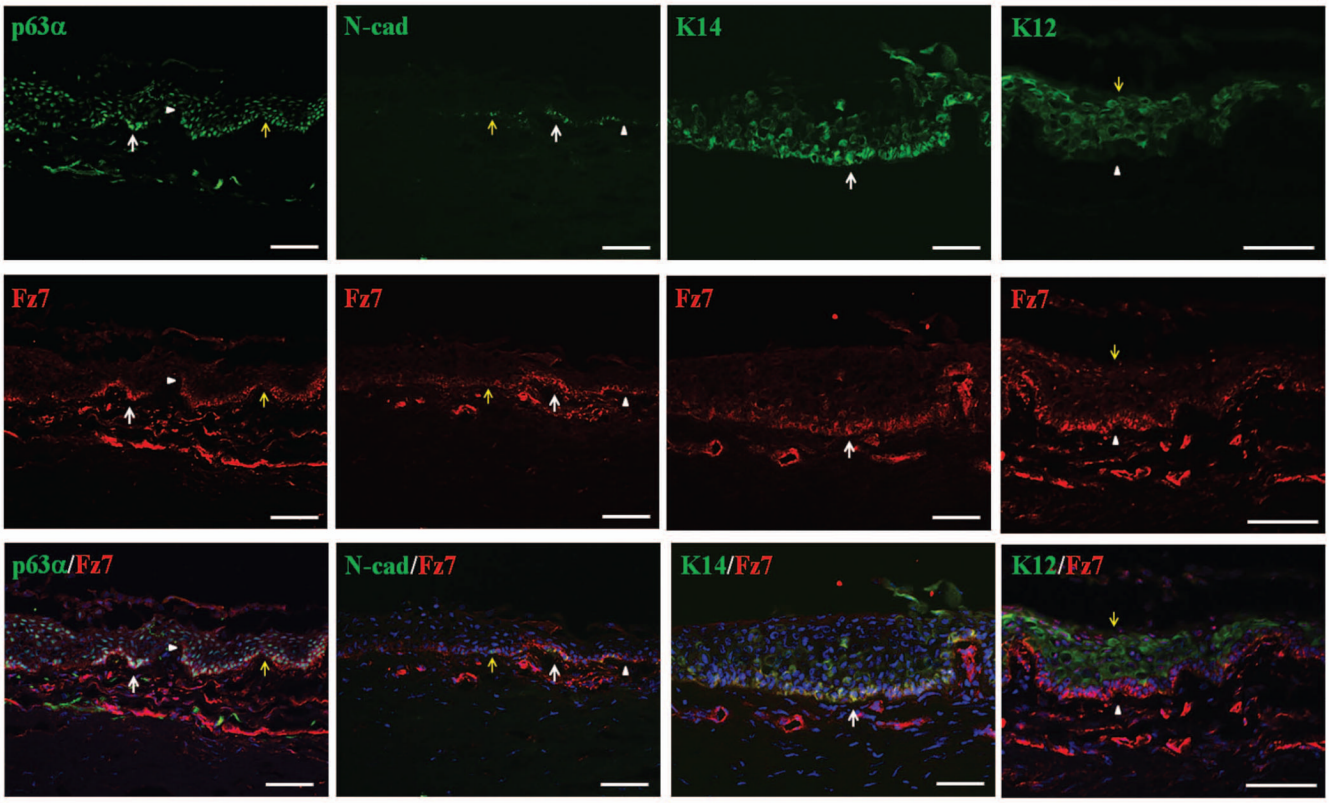

Figure 2.

The expression of Fz7 co-localized with putative LSC markers and the maturation marker K12 in human limbus by immunohistochemistry. Majority of the cells expressing high level of Fz7 colocalized with p63α, N-cadherin, and K14 and lack of K12 expression. White arrows indicate the basal epithelial cells strongly expressing both Fz7 and the putative stem cell marker. White arrowheads indicate the cells expressing a high level of Fz7 but low level of other cell marker. Yellow arrows indicate the cells expressing a high level of putative stem cell marker but low level of Fz7. Scale bar represents 50 μm.

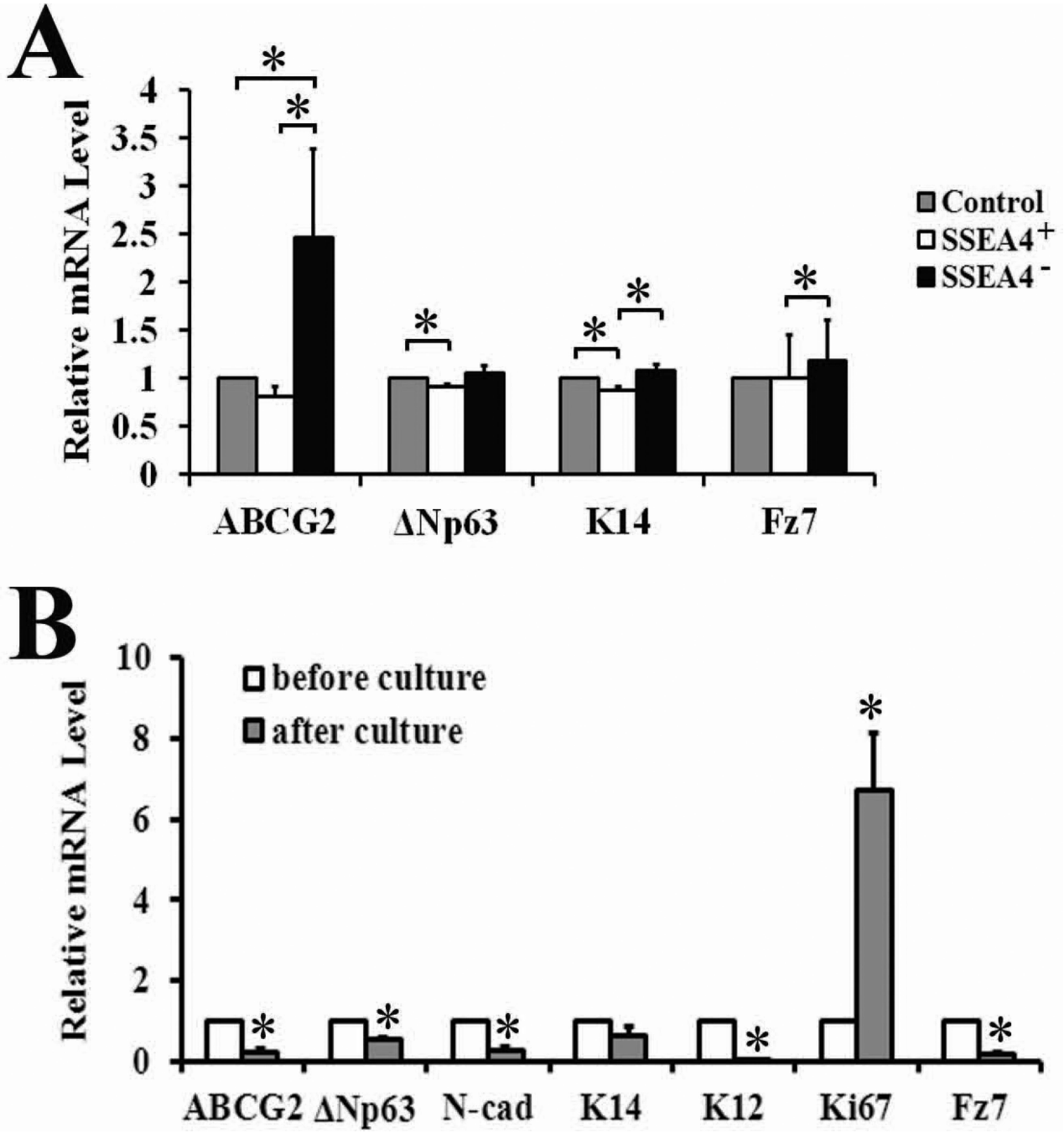

Figure 3.

The expression level of Fz7 was higher in the enriched LSC population. (A) The mRNA level of Fz7 was higher in the SSEA4^{-} LSC enriched population by qRT-PCR. *:$p<0.05$. (B) LSCs cultured on 3T3 feeder cells showed a decreased stem cell phenotypes and the expression of Fz7 decreased as well. *:$p<0.05$. Error bar represents S.E.M.

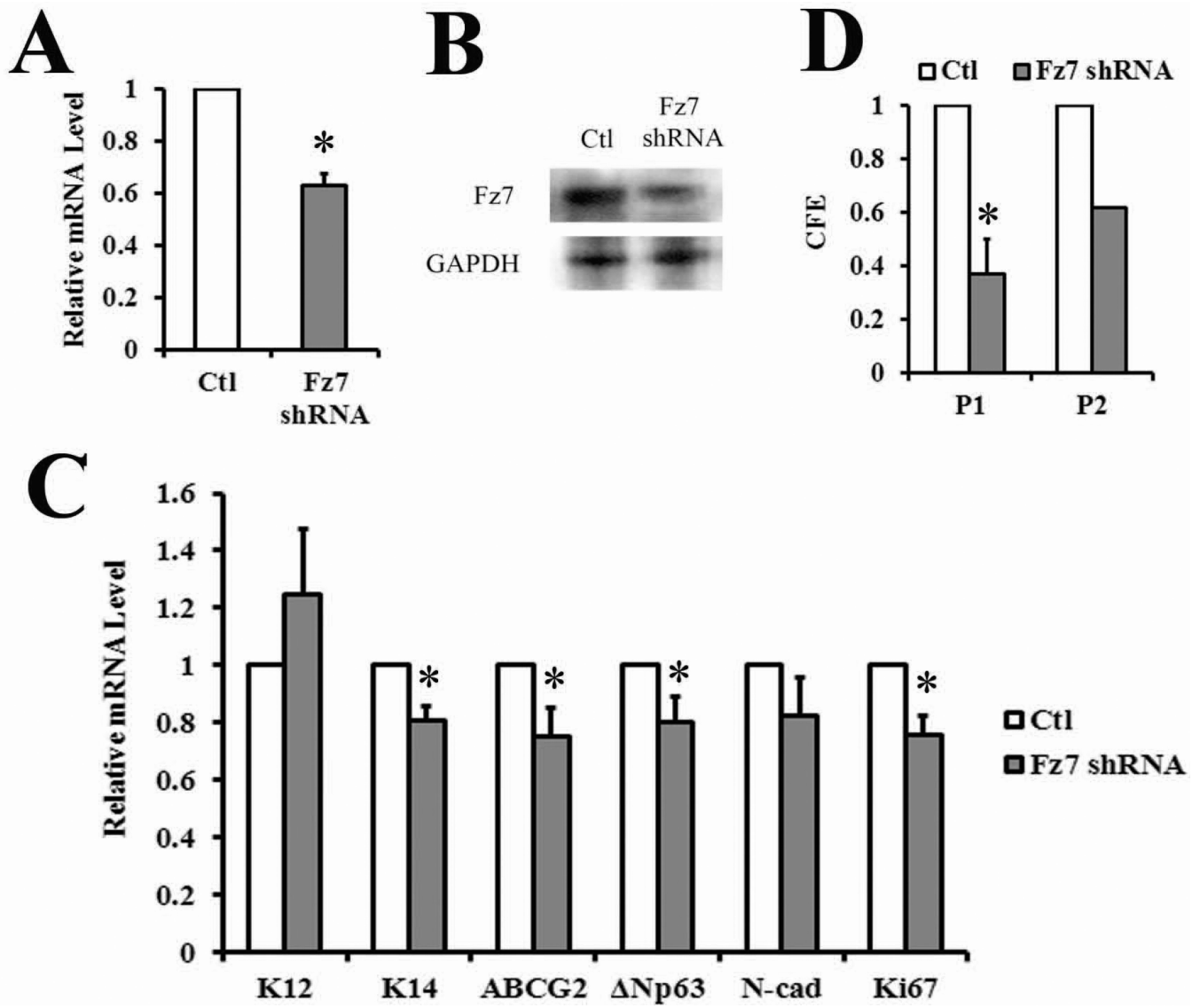

Figure 4.

(A) The mRNA level of Fz7 was knocked-down in LSCs ($Fz7^{KD}$) with Fz7 shRNA. (B) The protein level of Fz7 was lower in the $Fz7^{KD}$ LSCs by Western blot. (C) Knocked-down expression of Fz7 led to the decreased expressions of putative stem cell markers and proliferation marker through qRT-PCR. (D) Knock-down of Fz7 lead to a reduced colony forming efficiency. The transfected cells were passaged once (P1) or twice (P2) to form colonies. The CFEs for P1 and P2 were plotted. *$p<0.05$ Error bar represents S.E.M.

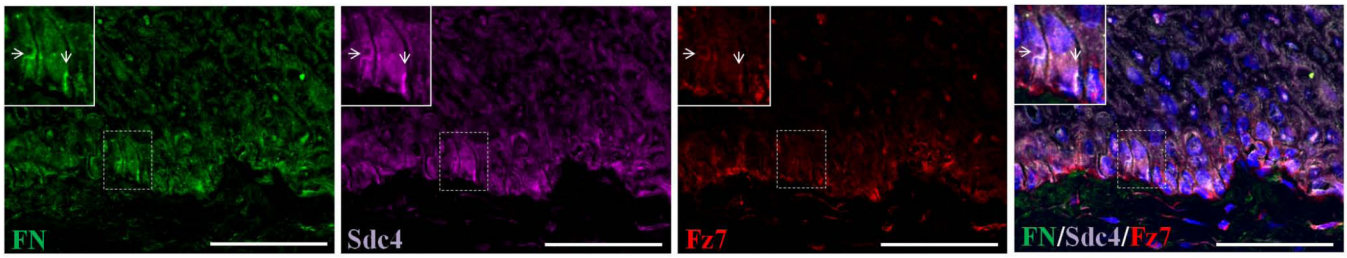

Figure 5.
The expression of Fz7 co-localized with Syndecan 4 (Sdc4) and fibronectin (FN) in some cells at the basal layer of human limbal epithelium. White arrows indicate the site where Fz7 co-localized with Sdc4 and FN between two adjacent basal epithelial cells. The insert shows the enlarged box area. Scale bar represents 50 μm.

Table 1

Primers in qRT-PCR

	Forward primer (5’-3’)	Reverse primer (5’-3’)
Fz1	AGACCGAGTGGTGTGTAATGA	AAATACTGTGAGTTGGCTTCGAT
Fz2	CGTGCCGCTCTATCTGTGAG	CCGTCCTCGGAGTGGTTCT
Fz3	GCTATGACTTGGATTGTCTTCTCT	AAGTGTGACACGTCCATATTCCAT
Fz4	TGTGTCTTTCAGTCAAGAGACGC	GGTTGTGGTCGTTCTGTGGT
Fz5	GCGCTTCTTCCTATGCTCTATG	GCTGCGGTTGTAATCCATGC
Fz6	GCATTTGTACCAACCTGCATAGA	TCTGTCACATTCAAGCTCCTCA
Fz7	CCGTACCACGGAGAGAAGG	GCGGAGTTCGGGAGAACAC
Fz8	TACAACCGCGTCAAGACAGG	CCATGTCGATAAGGAAGGTGGAG
Fz9	GCGGCACCAACACAGAGAA	AGACATAGCAAACGATGACGC
Fz10	GCAAGGACATCGGCTACAACA	GATGGGGGTAGAGACCTGCT
ABCG2	AACCTGGTCTCAACGCCATC	GTCGCGGTGCTCCATTTATC
ΔNp63	TTGTACCTGGAAAACAATGCC	TGCTGGTCCATGCTGTTCAG
N-cad	AGCCAACCTTAACTGAGGAGT	GGCAAGTTGATTGGAGGGATG
K14	GACCATTGAGGACCTGAGGA	ATTGATGTCGGCTTCCACAC
K12	CCAGGTGAGGTCAGCGTAGAA	CCTCCAGGTTGCTGATGAGC
Ki67	CTTTGGGTGCGACTTGACG	GTCGACCCCGCTCCTTTT
GAPDH	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG

Table 2

Primary and Secondary Antibodies

	Cat. No.	Company	Dilution
Fz1	BAM11201	R&D Systems	1:100
Fz4	MAB194	R&D Systems	1:200
Fz7	MAB1981	R&D Systems	1:200
P63α	4892S	Cell Signaling	1:100
N-cadherin	Sc-8424	Santa Cruz Biotechnology	1:200
K14	K14 Ab (Clone LL002)	NeoMarkers/Fisher Scientific	1:2
K12	Sc-25722	Santa Cruz Biotechnology	1:100
Syndecan-4	Ab24511	Abcam	1:20
Fibronectin	Ab23516	Abcam	1:20
Alexa Fluor donkey anti-rat	A21208	Invitrogen	1:500
Alexa Fluor donkey anti-mouse	A10036	Invitrogen	1:500
Alexa Fluor donkey anti-rabbit	A10040	Invitrogen	1:500
Alexa Fluor goat anti-rat	A11081	Invitrogen	1:500
Alexa Fluor donkey anti-sheep	A11015	Invitrogen	1:500
Alexa Fluor goat anti-rabbit	A21245	Invitrogen	1:500